UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS

# “EFECTO DE LA INSULINA SOBRE EL PERFIL PROTEÓMICO Y LA PARTICIPACIÓN DE LA VÍA PI3K/TOR EN EJES EMBRIONARIOS DE MAÍZ (*Zea mays* L.)”

**TESIS**

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PRESENTA:

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**Resumen.**

La insulina es una hormona de naturaleza peptídica que regula diferentes aspectos metabólicos en los animales. La principal función conocida de esta hormona es la regulación de la homeostasis de glucosa en sangre. Sin embargo, sus funciones abarcan aspectos metabólicos como: La estimulación de la glucogenogénesis, Inhibición de la glucogenolisis, promoción de la glucólisis, la síntesis de ácidos grasos y de proteínas. Este último proceso es mediado por la vía de señalización PI3K-TOR, donde TOR (Target Of Rapamycin) se conoce como el regulador maestro del crecimiento celular. Esta vía de señalización induce la biogénesis de ribosomas, aumentando la capacidad de síntesis de proteínas en la célula; esto conlleva a un aumento en la biomasa y por lo tanto a un crecimiento celular. La vía PI3K-TOR se encuentra conservada desde levaduras hasta plantas. En este último apenas se ha comenzado a entender los mecanismos a través de los cuales TOR regula el crecimiento, el cual es modulado en respuesta a factores externos (abióticos) e internos como los reguladores de crecimiento o fitohormonas. Se ha establecido una interacción entre la vía PI3K-TOR con las auxinas, implicadas en todos los aspectos del crecimiento y desarrollo vegetal. Por otro lado, desde hace algunos años se ha reportado que el maíz es capaz de producir un péptido similar a la insulina (ZmIGF) estructuralmente, que al igual que la insulina animal estimuló un aumento en el porcentaje de germinación e indujo el crecimiento vegetal. Recientemente se ha reportado que este ZmIGF es capaz de inducir la toma de glucosa del medio por adipocitos de rata. Además, se determinó la presencia de un receptor para dicho péptido a través del cual tanto el ZmIGF como la insulina podrían ejercer su acción dentro de la célula. Los datos antes mencionados muestran que tanto el ZmIGF como la insulina tienen un papel en la regulación del crecimiento celular vegetal y que parte de esta respuesta es a través de la vía de señalización PI3K-TOR. Sin embargo, se desconocen aspectos generales de la regulación por estos péptidos, por lo que en este trabajo se abordó este problema dando un enfoque proteómico de la respuesta a la insulina en ejes embrionarios de maíz durante la germinación. Los resultados obtenidos en el presente estudio nos muestran que la insulina promueve la producción de proteínas involucradas en aspectos metabólicos como la Gliceraldehido-3-fosfato deshidrogenasa y la Enolasa; por otro lado, promueve la degradación proteínas de almacenaje que funcionan como reserva de material durante la germinación, como las Globulinas y la Vicilina. Proteínas que participan en el proceso de germinación como las proteínas de embriogénesis tardía también fueron reguladas en respuesta a insulina. También se modifico el patrón de expresión de algunas proteínas que participan en respuesta a estrés abiótico; sin embargo, estas proteínas han sido reportadas como factores importantes para el proceso de germinación. Además, dado que se ha reportado que la insulina ejerce su efecto

de promoción del crecimiento a través de vía PI3K-TOR, usamos la rapamicina para inhibir la actividad de TOR. Los resultados muestran que la expresión de las proteínas antes mencionadas no a través de la activación de TOR. Estos datos indican que el efecto de la insulina sobre el maíz consiste en inducir, estimular y mantener el proceso de germinación. **Palabras clave**: insulina, mTOR, Señalización, Maíz, Perfil proteómico

**Summary.**

Insulin is a peptidic hormone that regulates several metabolic processes in animals. Glucose homeostasis is the most studied among these functions. Nonetheless, some other important functions of insulin are: Glycogenesis stimulation, glycogenolisis inhibition, glycolysis induction, stimulation of fatty acids synthesis, and translation promotion. Protein synthesis is mainly regulated by Target Of Rapamicyn (TOR), this protein has been proposed as the master regulator of cell growth. The pathway PI3K-TOR signaling induces ribosome biogenesis and, through this cell biomass is increased, promoting cell growth. This pathway is conserved from yeast to plants. In plants, the mechanisms involved in TOR signaling are scarcely understood. Plant growth is modulated in response to a several stimuli such as light, heat, and nutrient availability. Phytohormones play a crucial role in plant growth regulation, a cross regulation between TOR signaling and auxins, the most studied phytohormone, has been reported. On the other hand, regulators from peptidic nature have been reported in maize: an Insulin like Growth Factor (ZmIGF) that regulates germination and plant growth. This ZmIGF has been proven to be functional in rat adipocytes, capable of induce the uptake of glucose from the medium. Additionally, a putative receptor for this peptide and insulin has been reported in maize callus tissue, which could be the starting point of signaling induced by these peptides. All data above shows that ZmIGF and insulin modulates germination and plant growth trough similar mechanism to that in animals, probably trough the TOR signaling pathway. However, general aspects about insulin action and function in plants remain unknown, in this work we used a proteomic approach during maize germination as an attempt to unveil the proteins involved in response to insulin. Results show a modulation on metabolic proteins such as glyceraldehyde-3-phosphate dehydrogenase, and enolase1. On the other hand, insulin regulates the degradation of proteins that function as storage material during germination such as globulins and vicilin. Late embryogenesis proteins were also modulated by insulin. Some abiotic stress related proteins were modulated by this peptide, however, these proteins have been reported as important during the germination process, as an independent role from that to abiotic stress response. As insulin action in maize has been related to TOR signaling pathway, we used rapamicyn in order to address if these protein expression patterns were modulated trough this pathway, results showed that rapamycin had no effect on protein expression patterns, concluding that there is no participation of TOR in insulin

response observed in the protein expression pattern. These results show that insulin action on plant is directed to stimulate and enhance the germination process.

# I. INTRODUCCIÓN

## 1.1. Insulina

En la segunda década del siglo XX se dio el que podría ser el descubrimiento científico más importante en el campo de la medicina, la insulina. Antes del descubrimiento de esta hormona, las personas que padecían diabetes estaban condenadas a un proceso largo y doloroso que terminaba irremediablemente en la muerte. A pesar de que la enfermedad era fácilmente diagnosticada, no existía una cura para detener el avance de la enfermedad. Los primeros acercamientos hacia la etiología de la enfermedad condujeron al hígado como uno de los órganos involucrados en este proceso. Sin embargo, los pacientes diabéticos a los que se les realizaba la autopsia mostraban un daño significativo al páncreas. Estos hallazgos condujeron a investigar si el retirar el páncreas de perros podía aportar información sobre las consecuencias de este proceso quirúrgico. Esta cirugía se logró con éxito en 1889, observando que los perros sobrevivientes presentaban síntomas de diabetes. Entonces se empezó a usar el extracto de páncreas como terapia en los pacientes diabéticos sin resultados satisfactorios en la mayoría de los casos. Posteriormente un grupo de investigadores canadienses: Frederick Grant Banting, Charles Herbert Best, James Bertram Collip y John James Rickard Macleod; mejoró la preparación los extractos pancreáticos, para que fueran seguros y eficaces en los pacientes. Después de varios intentos fallidos, obtuvieron extractos etanólicos, de páncreas. El etanol produjo un precipitado que fue probado en un joven de 14 años con diabetes, el nuevo extracto pancreático redujo los niveles de glucosa en la sangre del joven y disminuyeron los cuerpos cetónicos presentes en la orina, lo que es un síntoma común de los pacientes con esta enfermedad. El descubrimiento fue anunciado en la reunión anual de la Association of American Physicians un 23 de mayo de 1922. El siguiente año Banting y Macleod fueron galardonados con el premio Nobel de Medicina por el descubrimiento (Rosenfeld, 2002; Karamitsos, 2011).

## 1.2. Procesos metabólicos regulados por la insulina.

La insulina fue la primera proteína en ser completamente secuenciada, hallazgo que mereció el premio Nobel de Química en 1955 para Frederick Sanger (Stretton, 2002). La estructura de la insulina consiste en dos cadenas peptídicas, A y B, unidas mediante puentes disulfuro. A pesar de esto, la insulina no se sintetiza como dos cadenas independientes que se unen más tarde. Después de la traducción del ARNm de la insulina, se obtiene una sola cadena peptídica denominada preproinsulina la cual se somete a una rápida escisión enzimática para generar proinsulina, la cual está conformada por las cadenas A y B unidas por un péptido denominado C (**Figura 1.1**). La proinsulina se empaqueta en pequeños gránulos dentro del complejo de Golgi, para posteriormente migrar hacia la superficie celular. Durante la maduración de estos gránulos las proteasas se encargan de separar el péptido C, dejando la insulina en su forma activa (cadenas A y B unidas por puentes disulfuro). La insulina forma microcristales alrededor de iones de zinc dentro de los gránulos de secreción, produciendo hexámeros que se separan rápidamente después de la liberación. El aumento de la glucosa intracelular desencadena la secreción de insulina mediante la activación de la glucocinasa seguido de un aumento de ATP intracelular, lo que resulta en el cierre de canales de potasio sensibles a ATP. Esto provoca la despolarización de la membrana de las células beta y la afluencia de iones de calcio, lo que lleva a la fusión de los gránulos de insulina con la membrana celular liberando la insulina, péptido C y otras moléculas (Docherty & Clark, 1994; Henquin, 2000)

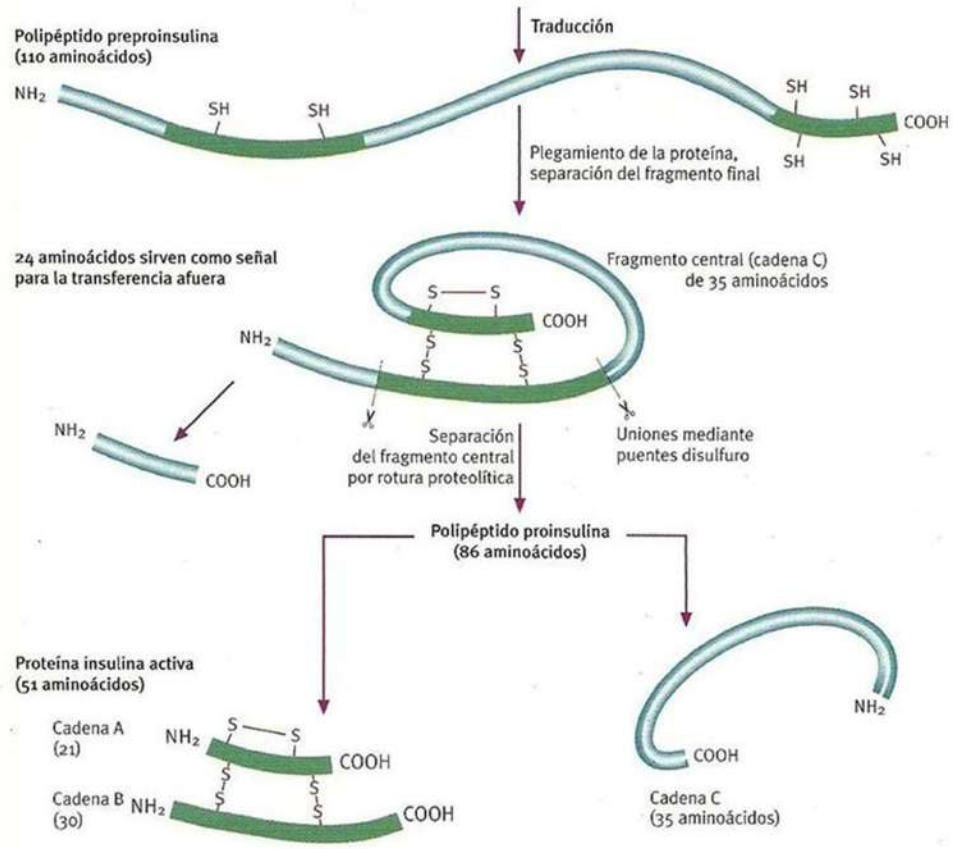


**Figura 1.1.** Síntesis y estructura de la insulina.

La insulina una vez liberada puede actuar en diferentes tejidos blanco (músculo esquelético, tejido adiposo e hígado) (**Figura 1.2**) regulando diversos aspectos fisiológicos como: metabolismo de carbohidratos, de ácidos grasos y de proteínas. Tanto en músculo esquelético como en tejido adiposo los efectos de la insulina son similares, en estos tejidos el metabolismo de la glucosa se ve afectado, al estimularse la glucólisis y la síntesis de glucógeno (glucogenogénesis)(Saltiel & Kahn, 2001; Zhang & Liu, 2014).

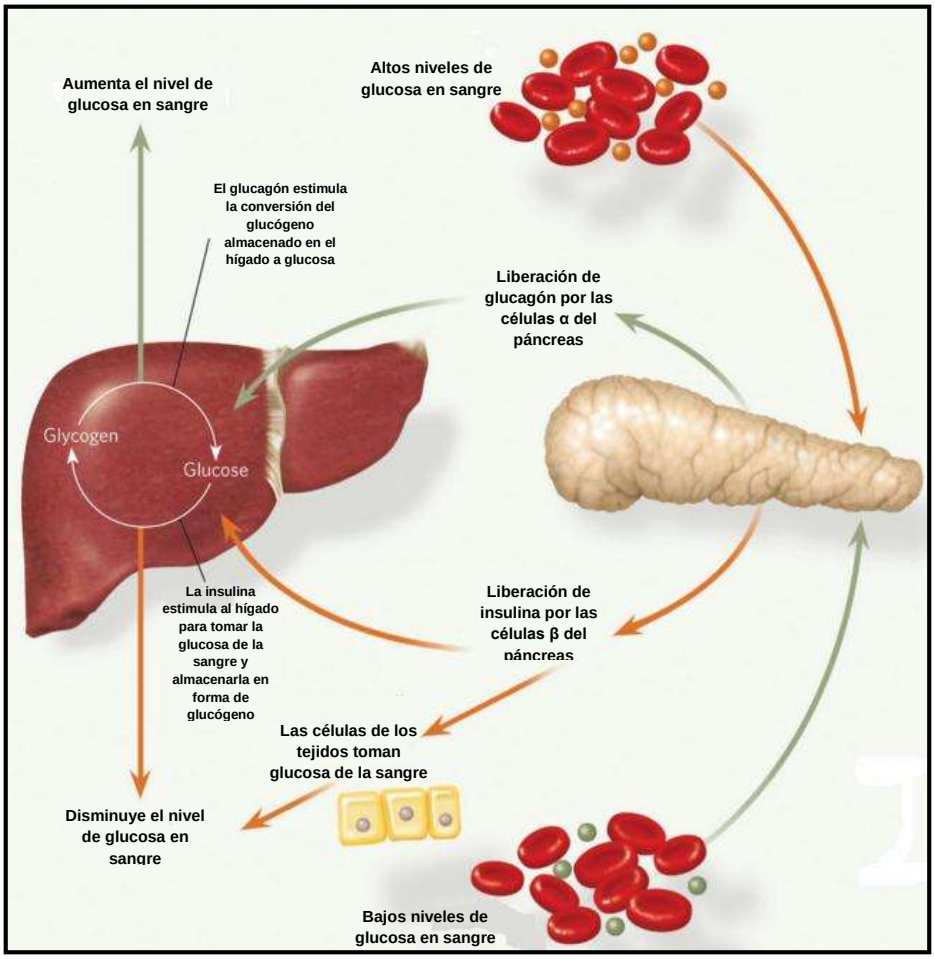


**Figura 1.2.** Aspectos fisiológicos de la insulina.

El incremento en la glucólisis se debe a un aumento en el ingreso de glucosa a la célula, la cual es fosforilada por la hexocinasa como primer paso de la vía. Esta vía metabólica consta de dos etapas: i) las primeras cinco reacciones que resultan en la formación de dos moléculas de gliceraldehído-3-fosfato y se consumen dos moléculas de ATP; ii) las subsecuentes cinco reacciones en donde el producto final son dos moléculas de piruvato, en esta fase se producen cuatro moléculas de ATP por lo que la producción neta es de dos moléculas de ATP por molécula de glucosa. El piruvato es entonces oxidado por el complejo enzimático piruvato deshidrogenasa, generando ácetil coenzima A (CoA). Esta reacción es un punto de control importante en el metabolismo de la glucosa ya que la ácetil CoA puede ser usada para generar energía liberando $CO_2$ en musculo esquelético o para la síntesis de lípidos en tejido adiposo e hígado (Lenzen, 2014).

El metabolismo de glucógeno es regulado por la glucógeno sintasa y la glucógeno fosforilasa. La primera es activada por la presencia de glucosa-6-fosfato para iniciar la síntesis de glucógeno. La segunda es activada por AMP para iniciar la degradación de glucógeno, y es inhibida por ATP. En presencia de insulina se activa la glucógeno sintasa debido al incremento de glucosa dentro de la célula, en ausencia de ésta hormona hay bajos niveles energéticos (aumentan los niveles de AMP) y la degradación de glucógeno se activa (Hems & Whitton, 1980; Saltiel & Kahn, 2001) .

El hígado, a diferencia de los otros tejidos, es capaz de degradar la glucosa (glucólisis) y de sintetizar glucosa (glucogenogénesis). La glucogenogénesis es el proceso mediante el cual se sintetiza glucosa a partir de precursores como: piruvato, lactato, glicerol, aminoácidos (excepto lisina y leucina) y todos los compuestos intermediarios del ciclo de Krebs. El proceso de glucólisis y glucogenogénesis son regulados de forma reciproca, si la glucólisis se está llevando a cabo la glucogenogénesis es inhibida y si ésta ultima esta activa la glucólisis entonces se detiene (Hems & Whitton, 1980).

De tal manera que la insulina regula diversos procesos fisiológicos relacionados con el metabolismo de carbohidratos (homeostasis de glucosa, glucólisis, glucogenogénesis, glucogenogénesis y glucogénolisis), metabolismo de ácidos grasos (Lipólisis y lipogénesis), también regula la síntesis y degradación de proteínas así como la captación de iones (**Figura 1.3**).

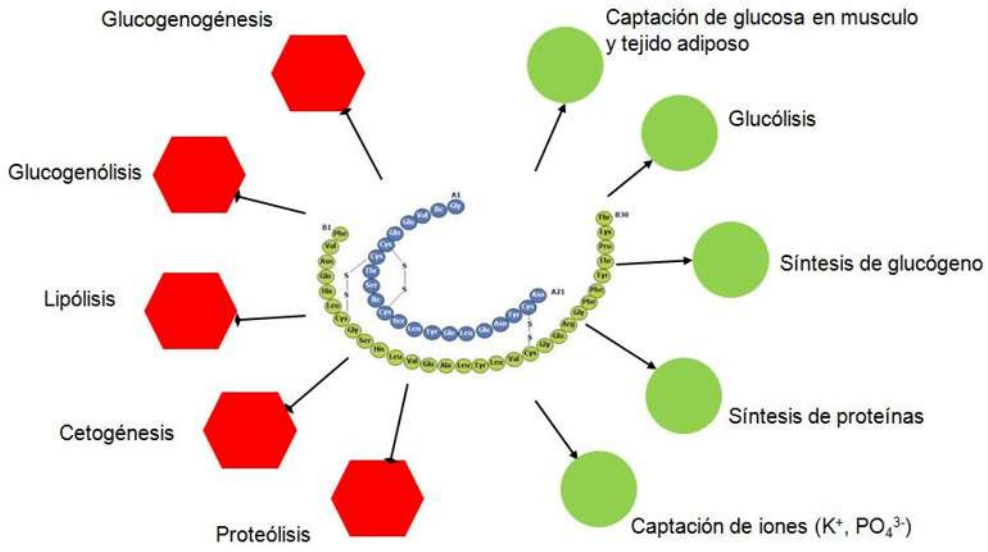


**Figura 1.3.** Procesos metabólicos regulados en respuesta a la insulina. En color rojo se muestran los procesos que se inhiben, mientras que en verde vemos aquellos que se inducen (Modificado Gaw et al, 2008).

## 1.3. Mecanismos de acción de la insulina.

Los mecanismos de acción modulados por la insulina involucran procesos de fosforilación y defosforilación de proteínas moduladoras de los aspectos metabólicos mencionados anteriormente, además de que las vías de señalización desencadenadas por la insulina activan o inactivan la expresión de genes involucrados en dichos procesos.

Como la gran mayoría de las hormonas y diversos estímulos extracelulares, la insulina se une a un receptor especifico anclado a la membrana celular. Al acoplarse la insulina a su receptor se inicia una cascada de eventos al interior de la célula. El receptor de insulina es miembro de una superfamilia de receptores de tipo tirosina cinasa (RTKs, por sus siglas en ingles), éste receptor consta de dos sub-unidades α extracelulares, que contienen el dominio de unión al ligando; y dos sub-unidades β transmembranales que contienen el dominio de tirosina cinasa. La insulina al acoplarse a las subunidades α del receptor, induce un cambio conformacional en las subunidades β que permite la auto-fosforilación de éstas

(Becker & Roth, 1990). Una vez activado el receptor, tiene como principal sustrato a los miembros de la familia de sustrato del receptor de insulina (IRS 1, 2, 3 y 4, por sus siglas en ingles). La fosforilación en tirosinas de estas proteínas IRS permite la unión de éstas con proteínas efectoras que contengan un dominio de unión a residuos de tirosina fosforilada denominado SH2 (dominio homologo de Src 2), una de estas proteínas efectoras es la PI3K (fosfatidilinositol 3-cinasa) la cual se encarga de fosforilar al fosfatidilinositol-4,5-difosfato ($PIP_2$) para generar fosfatidilinositol-3,4,5-trifosfato ($PIP_3$), este proceso puede ser inhibido de manera natural por la fosfatasa homologo e tensina (PTEN) y mediante el uso de compuestos químicos inhibidores de PI3K como la Wortmanina y el LY294002 (Shepherd et al, 1998). La producción de $PIP_3$ crea sitios de reconocimiento para proteínas con dominios homólogos a pleckstrina (PH) tales como las proteínas de la superfamilia AGC (PKA, PKG y PKC) de serina/treonina cinasas, de especial importancia para la señalización por insulina son la proteína dependiente de fosfoinosítidos (PDK1) y la proteína cinasa B (PKB, también llamada Akt). Donde, Akt al unirse al $PIP_3$ sufre un cambio conformacional que permite a PDK1 fosforilarla. Una vez fosforilada Akt representa un punto de crucial importancia para la regulación de la señalización por insulina, esta proteína es capaz de fosforilar diferentes sustratos para dirigir la señal hacia diversos procesos celulares (Fayard et al, 2005).

Akt es la encargada de regular el proceso de entrada de glucosa a la célula mediante la inhibición por medio de fosforilación de la proteína activadora de la actividad de GTPasa de Rab denominada Sustrato de Akt de 160 KDa (AS160), Rab al ser activada promueve la reorganización del citoesqueleto, lo que permite la translocación de los transportadores de glucosa GLUT4 a la membrana celular. Por otro lado, la cinasa de la glucógeno sintasa-3 (GSK3) fue la primera proteína descrita como blanco de Akt, esta cinasa al ser fosforilada se inhibe su actividad hacia la glucógeno sintasa con lo que ésta última queda activa y aumenta la síntesis de glucógeno. Akt también regula la expresión de genes relacionados modulando la actividad de los factores transcripcionales FOXO, en los cuales la fosforilación inhibe su actividad, FOXO1 en hígado regula la expresión de genes

que participan en la gluconeogénesis y al ser fosforilado por Akt permite la interacción de éste con las proteínas 14-3-3 con lo que FOXO1 se mantiene en el citoplasma inhibiendo este proceso (Brazil et al, 2004).

Una de las funciones mejor conocidas de Akt es la regulación sobre la vía de señalización de la proteína blanco de la Rapamicina (mTOR, en mamíferos) a través de la cual se modula el crecimiento celular (Manning & Cantley, 2007). Río abajo de Akt se encuentra el complejo de esclerosis tuberosa (TSC1/TSC2) el cual funciona como proteína activadora de GTPasa (GAP). Este complejo, específicamente TSC2, al ser fosforilado por Akt (en los residuos S939, S981, S1130, S1132 y T1462) es inhibido, con lo que pierde su actividad hacia la GTPasa Rheb, manteniéndose esta última unida a GTP. Se ha establecido que Rheb interactúa directamente con el dominio de cinasa de mTOR y que se requiere que la GTPasa este unida a GTP para que pueda activar a mTOR (Dibble & Cantley, 2015).

La proteína mTOR forma dos complejos multiproteicos denominados mTORC1 y mTORC2, estos complejos son distintos tanto en componentes como en función (Wullschleger et al, 2006; Laplante & Sabatini, 2013). El complejo mTORC2 (formado por: mTOR, rictor, SIN1, DEPTOR y mLST8) es insensible a la Rapamicina y sus principales funciones reportadas son la reorganización del citoesqueleto de actina, fosforila proteínas cinasa de la familia AGC cinasas como SGK y Akt; además, recientemente se reportó que se encarga de la degradación de polipéptidos recién sintetizados (Oh & Jacinto, 2011). Por otro lado, el complejo mTORC1 (formado por: mTOR, raptor, mLST8, DEPTOR y PRAS40) ha sido ampliamente estudiado debido a su participación en la integración de diversas señales que derivan en la regulación de aspectos metabólicos como: síntesis de proteínas, de lípidos y autofagia (Wullschleger et al., 2006; Laplante & Sabatini, 2009). Gran parte de la información conocida sobre la función de este complejo proviene del uso del compuesto aislado de *Streptomyces hygroscopicus* denominado Rapamicina, este macrólido se une a la proteína de unión a FK506 de

12 KDa (FKBP12) con la cual forma un complejo ternario con mTOR inhibiendo su actividad (Ballou & Lin, 2008).

Río abajo de mTOR los blancos descritos más ampliamente son: la proteína de unión al factor de inicio de la traducción 4E (4EBP1) y la cinasa de la proteína ribosomal S6 (S6K) (M. Laplante & Sabatini, 2009); La primera al ser fosforilada por mTOR liberal factor de inicio de la traducción 4E (eIF4E) con lo cual se promueve la traducción CAP-dependiente. Por otro lado, la S6K requiere de una posterior fosforilación por PDK1 para ser activada, una vez activada tiene diversos blancos como: la proteína S6 del ribosoma, el factor de inicio de la traducción 4B (eIF4B), el sustrato del receptor de insulina 1 (IRS1), la cinasa del factor de elongación de la traducción 2 (eEF2K), la proteína pro-apoptótica BAD, incluso la misma mTOR (Ruvinsky & Meyuhas, 2006). A través de estas proteínas se regula la síntesis de proteínas con lo que se da un aumento en la biomasa induciendo así el crecimiento celular (**Figura 1.4**).

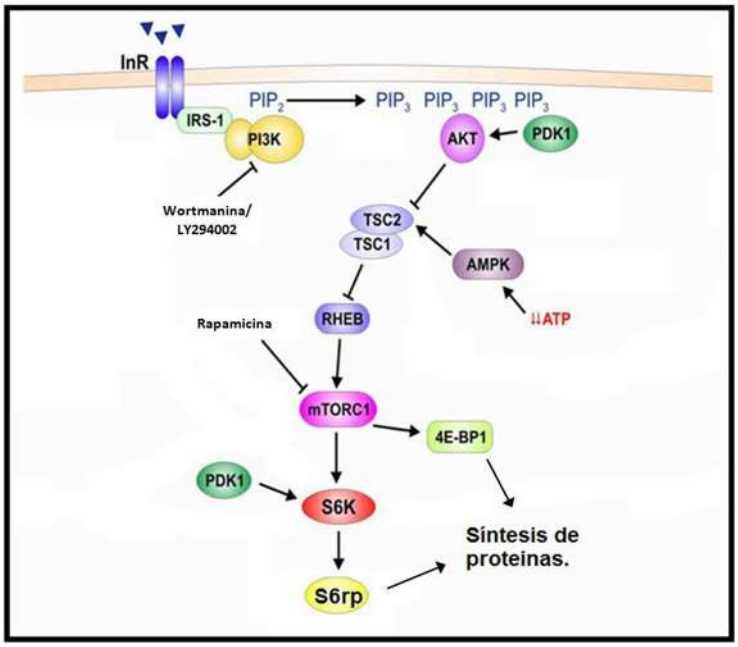


**Figura 1.4.** Vía de señalización de mTOR. Modificado de (Jastrzebski et al, 2007)

# II. ANTECEDENTES

## 2.1. Insulina en plantas.

Después del descubrimiento de la insulina en animales por el grupo de científicos canadienses (Banting, Best, Collip y Macleod), Collip y Best reportaron compuestos tipo insulina encontrados en diferentes plantas como: cebolla, lechuga, cebada, papa, arroz, entre otras. Collip realizo los aislados y los probó en perros a los que les había sido extirpado el páncreas, donde encontró que los niveles de glucosa disminuyeron a consecuencia de la administración de los extractos vegetales (Collip, 1923. Best & Scott, 1923. Best, 1924). Desde entonces, se han identificando péptidos similares a la insulina presentes en distintos tipos de organismos como: Escherichia coli, Tetrahymena pyriformis (LeRoith et al, 1985); y Neurospora crassa (Muthukumar & Lenard, 1991).

Sin embargo, desde entonces poco interés se puso en las plantas. Fue hasta la década de los 70's cuando surgió nueva información sobre el tema, Khanna y col. registraron una patente en los Estados Unidos para un método de extracción de insulina a partir de tejido vegetal. Siguiendo esa metodología se logró extraer fracciones con actividad similar a la insulina de animales sobre adipocitos aislados (Ng et al, 1986). Por esa misma década, se reportó el aislamiento, purificación y caracterización de un péptido similar a la insulina de espinaca y Lemna gibba (Colliersfi et al, 1987). En el final de la década de los noventa, tomado como antecedentes la presencia de péptidos con actividad de factor de crecimiento en plantas, se reportó el efecto de la insulina animal sobre cuestiones bioquímicas y fisiológicas del maíz. La insulina fue capaz de inducir la germinación, de aumentar la síntesis de proteínas ribosomales e inducir la fosforilación de la proteína ribosomal S6, lo que fue inhibido por Rapamicina y wortmanina, compuestos usados para identificar la vía de señalización involucrada en la respuesta a insulina en mamíferos (Sánchez de Jiménez et al, 1999). A partir de este descubrimiento, este grupo de trabajo ha reportado la presencia de un péptido de 20KDa similar a la insulina propio del maíz, este péptido es capaz de inducir la germinación y el crecimiento de plántulas; así como la fosforilación de la proteína S6 de la sub-unidad 40S del ribosoma y aumentar la síntesis de propeinas

ribosomales, todo esto de manera similar al efecto de la insulina animal reportado previamente por ellos (García Flores, 2001). Este descubrimiento indicó la presencia de un péptido similar a la insulina presente en el maíz que actúa como un factor de crecimiento en animales. Posteriormente se reportó la purificación de este péptido por cromatografía líquida de alta eficacia en fase reversa (RT-HPLC, por sus siglas en ingles) para purificar este péptido, donde se incluyó un análisis por dicroísmo circular que mostró una estructura de α-helice similar a la presente en la insulina y factores de crecimiento tipo insulina (IGFs) (Rodríguez-lópez, 2011).

Lo evidencia presentada por los grupos de trabajo mencionados anteriormente muestra que en plantas existe un péptido similar a la insulina tanto en estructura como en propiedades fisiológicas y bioquímicas. La insulina animal a servido como herramienta para el estudio de la respuesta a este péptido, usándola para identificar posibles vías de transducción de señales involucradas en la respuesta a esta hormona. Hasta la fecha la vía de señalización PI3K-TOR ha sido la más ampliamente estudiada como reguladora del crecimiento celular en respuesta a la insulina en plantas, específicamente en maíz. A continuación se hace una revisión de esta via de señalización en plantas y el papel de la insulina en su regulación.

## 2.2 La vía de señalización de TOR en plantas.

La vía de señalización de TOR se encuentra conservada desde levaduras hasta plantas, si bien no se conocen a fondo los mecanismos de regulación involucrados en plantas, se conocen los componentes esenciales de esta ruta de señalización sugiriendo que su funcionamiento es muy similar al reportado en mamíferos.

La activación de una vía de señalización inicia con la presencia de un efector, una molécula que al acoplarse a un receptor desencadena una serie de eventos intracelulares que detona una respuesta. En la ruta de TOR en plantas se ha demostrado que tanto la insulina animal como reguladores del crecimiento vegetal, como las auxinas; activan esta red de señalización (Sánchez de Jiménez et al.,

1999; García Flores et al, 2001; Beltrán Peña et al, 2002; Schepetilnikov et al., 2013). Los mecanismos exactos a través de los cuales tanto auxinas como insulina activa a TOR no quedan del todo claro. Para el caso de las auxinas una propuesta sobre la activación de TOR podría ser a través de la fosfolipasa D la cual es activada en respuesta a auxinas y produce acido fosfatídico, el cual funciona como segundo mensajero para activar a la proteína PDK1 la cual a su vez sería la encargada de fosforilar y activar a TOR (Bögre et al, 2013).

En el caso de la insulina o factores de crecimiento tipo insulina, se ha descrito recientemente un posible receptor para estos péptidos en maíz (Garrocho-Villegas et al, 2013); sin embargo no queda claro el posible segundo mensajero involucrado en desencadenar la señalización. El efecto de inhibidores como la wortmanina o el LY294002, indican que la proteína PI3K de plantas está involucrada en dicha respuesta (Lee et al., 2008; Liu et al, 2012; Takáč et al, 2013). Esto a pesar de que en plantas solo esta presente la PI3K de clase III, ya que en mamíferos existen las clases I, II y III; siendo la clase II la responsable en la señalización por insulina (Lee et al, 2010).

La señalización después de la PI3K no está del todo esclarecida, en plantas no se ha encontrado un ortólogo de la proteína Akt; una proteína de crucial importancia en la respuesta a insulina en mamíferos. Sin embargo, en respuesta a reguladores del crecimiento vegetal o la insulina, la proteína TOR es activada evidenciado por un aumento en la fosforilación de la proteína S6K que además correlaciona con un aumento en la fosforilación de la proteína S6 de la subunidad pequeña del ribosoma, indicando que los elementos importantes de la vía de señalización se encuentran conservados y que ésta regula la síntesis de proteínas ribosomales de manera similar a como se realiza en mamíferos (Dinkova et al., 2007; Garrocho-Villegas et al, 2012; Xiong & Sheen, 2014; Rexin et al, 2015) (**Figura 2.1.**)

Esta vía de señalización en plantas ha sido estudiada gracias al uso del inhibidor del complejo 1 de TOR en mamíferos, la Rapamicina. Este compuesto ha generado una serie de controversias en plantas debido a la aparente resistencia

de la planta *Arabidopsis thaliana* a los efectos de éste (Sormani et al., 2007). Sin embargo, diversos reportes en maíz han indicado que la Rapamicina es una herramienta confiable para el estudio del papel de TOR en diversos aspectos del desarrollo y crecimiento vegetal (García Flores et al, 2001; Reyes de la Cruz et al, 2004; Dinkova et al., 2007; Garrocho-Villegas et al, 2012). Recientemente en Arabidopsis se ha reportado la sensibilidad a la Rapamicina, usando concentraciones 10 veces más elevadas a las usadas anteriormente en ésta planta y a las usadas comúnmente en mamíferos (Xiong & Sheen, 2012), mostrando que la Rapamicina es una herramienta valida y muy útil para estudiar el papel de TOR en plantas.

La evidencia muestra entonces, que la insulina puede acoplarse a un receptor en células vegetales y que a partir de ahí puede desencadenar la señalización seguida de una respuesta para regular el crecimiento y desarrollo vegetal.

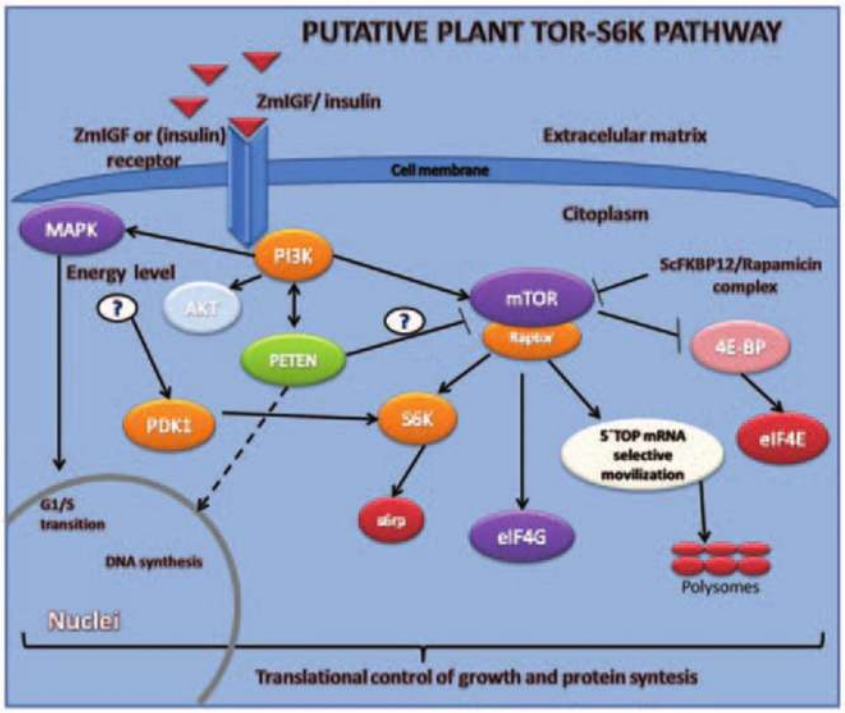


**Figura 2.1.** Señalización de TOR en plantas. Imagen tomada de (Garrocho-Villegas & Sánchez de Jiménez, 2012)

## III. Justificación.

La insulina es una hormona que regula diversos aspectos metabólicos en mamíferos. En plantas, por otro lado, se ha descrito la presencia de un péptido similar a la insulina que regula ciertos aspectos del crecimiento y desarrollo vegetal a través de vías de señalización conservadas como la ruta PI3K-TOR. Sin embargo, siguen sin ser claras las funciones y el rol de un péptido con estas características en el desarrollo de las plantas. Los antecedentes presentados anteriormente lo ubican como un regulador y promotor de la germinación de maíz, siendo relacionado con la regulación de la síntesis de proteínas a través de la vía mencionada. Para comprender mejor el rol de dicho péptido en plantas, en este trabajo se usa un perfil proteómico de la respuesta a insulina en ejes embrionarios de maíz y de esta manera se espera obtener información sobre la respuesta general a un estimulo por esta hormona.

## IV. Hipótesis.

Un perfil proteómico de ejes embrionarios de maíz permitirá identificar elementos involucrados en la respuesta a insulina, así como el papel de la vía PI3K/TOR.

## V. Objetivos.

### 5.1. Objetivo general.

Caracterizar el efecto de insulina sobre el proteoma de ejes embrionarios de maíz y la participación de la vía PI3K/TOR.

### 5.2. Objetivos específicos.

- Determinar el efecto de la Insulina sobre el proteoma de ejes embrionarios de maíz.
- Determinar la participación de vías de señalización PI3K/TOR en el efecto de la Insulina sobre el proteoma de los ejes embrionarios de maíz.

# VI. RESULTADOS

# Insulin induces changes in metabolism-related proteins during maize germination

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Key words: Germination, Insulin response, TOR signaling

Abbreviations: **TOR**, Target of Rapamycin; **IGF**, Insulin-like Growth Factor; **ZmIGF**, maize Insulin-related Growth Factor; **Rap**, rapamycin; **S6rp**, S6 ribosomal protein; **S6K**, S6rp Kinase

Total number of words: 4908

**Abstract**

Insulin regulates a wide range of metabolic processes in mammals, such as homeostasis and the breakdown of glucose among others. Recently, an insulin-related growth factor in maize (ZmIGF) and a possible receptor for this growth factor has been reported. This peptide exerts effects on plant growth and promotes germination by activating target of rapamycin (TOR) signaling pathways, which is similar to insulin response in mammals. In this study, we analyzed the insulin response in maize embryos using a proteomic approach. Our results indicate that insulin modulates the expression of proteins involved in processes such as storage protein degradation, protein processing, redox and desiccation stress, and glucose metabolism. The involvement of TOR signaling pathways was analyzed using the TOR inhibitor rapamycin. The results showed that the modulation of these proteins by insulin is independent of the TOR pathway. These results indicate that insulin promotes changes in metabolism-related proteins to ensure successful germination in maize.

## Statement of significance of the study

In a molecular level, several cellular processes are evolutionarily conserved among eukaryotes. Signal reception and transduction of growth factors, particularly, insulin and insulin-like growth factors (IGFs) are well documented, and several signal transduction pathways have been elucidated in non-plant eukaryotes. Nonetheless, in plants the mechanism of action of peptidic growth regulators is scarce. Insulin effect on carbohydrates and lipid metabolism are extensively documented in non-plant organisms, however, no reports have been documented related to insulin action on metabolic pathways in plants, although its effect on germination and growth has been reported. Target of rapamycin (TOR) protein has emerged as a common component of signaling pathways involved in the sequel of several growth factors, mainly insulin and IGFs, in eukaryotes its has been pointed out as cell growth regulator. In plants, play a central role in embryogenesis, meristem activation, root and leaf growth, flowering, senescence, and life span determination. Our results about insulin effect on proteome of maize embryos indicates that insulin action mechanism is through regulating expression of proteins involved in carbohydrate metabolism, utilization of storage nutrients and protein folding, as occurs in non-plant organisms. Interestingly, this action mechanism is TOR-independent.

## Introduction

Insulin is a critical hormone in mammals that regulates a wide range of metabolic processes, such as glucose uptake and breakdown as well as fatty acid and protein synthesis [1]. There have been reports of the effects of insulin on plant growth since its isolation in the early 1920s [2], but it was not until the last few decades that the mechanisms of the effects of insulin on plant growth have been investigated at a biochemical level, generating new information about the effects of insulin in plants. Sanchez de Jimenez *et al*. found that bovine insulin accelerates maize germination, seedling growth, and protein synthesis in a rapamycin-sensitive manner [3]. They reported that the isolation and characterization of a peptide from maize (ZmIGF) recognized by antibodies against bovine insulin showed insulin-like growth factor (IGF) activity, inducing S6 ribosomal protein phosphorylation, and enhanced ribosomal protein synthesis [4]. These effects were exerted through the target of rapamycin protein (TOR), as determined by analyzing the sensitivity of the effects to rapamycin [4, 5]. In non-plant eukaryotes, TOR is found in at least two distinct complexes, TOR complex1 (TORC1) and TORC2. The rapamycin-sensitive mammalian mTORC1, contains mammalian TOR (mTOR), mLST8, and RAPTOR. This complex responds to amino acids, glucose, insulin, and growth factors to control proliferation and temporal cell growth by promoting anabolic processes (translation, transcription, and ribosome biogenesis) and negatively regulates autophagy. Most of the functions of mTORC1 are mediated through translational control by its substrates, S6 kinase (S6K) and eukaryotic translation initiation factor 4E binding protein1 (4E-BP1). By contrast, the rapamycin-insensitive mTORC2,

controls spatial cell growth by regulating cytoskeleton structure and polarity, as well as glycolysis, glycogenesis, lipogenesis, and gluconeogenesis, via Akt phosphorylation [6, 7]. mTORC2 contains mTOR, mLST8, and RICTOR. The precise compositions of the TOR kinase complexes have not been characterized in plants. However, some mTORC1 components and downstream effectors have been identified in photosynthetic eukaryotes from *Chlamydomonas reinhardtii* to plants, including Arabidopsis RAPTOR1/RAPTOR2, LST8-1/LST8-2, S6K1/S6K2, ribosome protein small subunit6 (RPS6A/B), type 2A-phosphatase-associated protein (TAP46), and ErbB-3 epidermal growth factor receptor binding protein (EBP1) [6, 7]. In addition to its similar rapamycin sensitivity and its role in modulating endogenous S6K phosphorylation and nutrient-dependent growth, endogenous Arabidopsis TOR phosphorylates human 4E-BP1, indicating the existence of a functionally conserved TORC1 in plants. By contrast, there is no evidence of a plant TORC2 to date, because specific components of this complex, such as RICTOR, seem to be absent from the genomes of photosynthetic organisms [6, 7]. In a previous study, a complete analysis of the TOR-S6K signaling pathway was performed, and the effect of insulin on maize embryonic axes was evaluated; the regulation of this pathway in response to ZmIGF or insulin in maize was similar to that observed in response to insulin in mammals [8]. Since insulin is a regulator of cell cycle in mammals, the association of this hormone with ZmIGF in the cell cycle in maize was evaluated by observing increases in DNA synthesis and mitotic index, selective translation of D-type cyclins, and proliferating cell nuclear antigens [9]. Purification of ZmIGF was performed using reverse phase-high performance liquid chromatography; a 5.7 KDa peptide with an α-helix

structure similar to that of animal insulin or IGF was obtained [8]. Recently, TOR signaling pathway activation, DNA synthesis stimulation, and selective translation of mRNAs was observed in response to insulin or ZmIGF by using fast and slow models of growing maize calli [10]. Interestingly, a sequence for the ZmIGF β-chain, showing similar structural arrangement to human insulin or IGFs, and a 55-kDa insulin-like receptor immunolocalized on the cell membranes in maize plants was reported [11]. The use of rapamycin to evaluate TOR signaling has been an important tool because protein synthesis, S6rp and S6K phosphorylation in maize are sensitive to this compound; moreover, recently, has also been reported that rapamycin inhibits TOR-S6K1 signaling and retards glucose-mediated root and leaf growth in *Arabidopsis* [12], which highlights the importance of this compound in elucidating plant growth mechanisms driven by TOR signaling pathways. Despite of all these information, the molecular mechanisms of insulin or ZmIGF and plant responses to these compounds are still poorly understood. Hence, the objective of this work was to assess the response to insulin during maize germination using a proteomic approach. The results presented here show that insulin modulates the expression of proteins involved in processes such as the degradation of storage proteins, protein processing, protection of maize embryos from redox and desiccation stresses, and interestingly, glucose metabolism. The involvement of the TOR signaling pathway was analyzed using the TOR inhibitor, rapamycin. Our results show that modulation of protein expression by insulin takes place in a TOR-independent fashion. Taken together, our findings suggest that insulin plays a role

in improving germination and plant growth by inducing changes in metabolism-related proteins.

## Materials and methods

### *Plant material*

Seeds and embryonic axes from maize (*Zea mays* L. cv Chalqueño) were used in all experiments. The axes were manually dissected from the seeds and immediately used for treatments.

### *Germination conditions and treatments*

Two sets of 125 seeds were surface disinfected using ethanol (70%) for 5 min, washed three times with distilled water, and placed in sterile water-imbibed cotton beds for 22 h at 24 ± 2°C in darkness. For each treatment, 50 embryonic axes were incubated in liquid Murashige-Skoog (MS) medium [13] containing either 200 µU (1.23 nM) of human insulin (regular humulin, Lilly, LLC, IN, USA), 200 µU of human insulin plus 100 nM rapamycin (LC Laboratories, Woburn, MA, USA), or 100 nM rapamycin alone. For wound treatment, the embryonic axes were cut longitudinally. Control treatment included an equal volume of methanol as a solvent used for rapamycin solution. After 2 h of treatment, the embryonic axes were washed twice with sterile water, and then frozen in liquid $N_2$ for protein extraction or stored at -80°C until use.

### *Protein isolation and 2D electrophoresis*

Excised embryos (250 mg) were ground in liquid nitrogen using a mortar and pestle and homogenized in 10 mL of buffer solution containing 50% [v/v] phenol, pH 8.8, 0.9 M sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.4% [v/v] 2-mercaptoethanol, 100 mM Tris-HCl, pH 8.8, EDTA, and protease inhibitors (Complete, Roche Molecular Diagnostics, Pleasanton, CA). The homogenate was agitated for 30 min at 4°C on a shaker. After a 15 min centrifugation at 10,000*g* and 4°C, the phenol phase was removed and proteins were precipitated with 5 volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at −80°C for 2 h. The protein pellet was recovered after a 10 min centrifugation at 10,000*g*, washed twice in 10 mL of 0.1 M ammonium acetate in 100% methanol, twice in ice-cold 80% acetone, and once in 70% ethanol. The pellet was dried for 5 min at room temperature and resuspended immediately in 1 mL of isoelectrofocusing (IEF) buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) Triton X-100, 50 mM dithiothreitol (DTT), and EDTA-free complete protease inhibitors. Insoluble matter was removed by centrifugation for 20 min at 18,000*g*. Protein concentration was determined in triplicates against a standard curve of bovine serum albumin (BSA) using a protein assay (Bio-Rad, Hercules, CA) based on the Bradford method [14]. Immobile DryStrips gels, non-lineal pH 3-10 (13cm) were rehydrated adding 150 µl of rehydration solution (7M urea, 2M thiourea, 4% CHAPS, 50mM hydroxyethyldisulfide, 2% IPG buffer pH 3-10) to IEF buffer containing protein sample (300 µg) for a total volume of 250 µL that were loaded onto DryStrip Reswelling Tray for 24 h. IEF was performed in an Ettan IPGphor 3 IEF system (GE healthcare) under the following conditions: 100 V for 100 Vh, 500 V for 500 Vh, and 8000 V for 99 kVh. Following IEF, the IPG strips were incubated in SDS

equilibration buffer (1.5 M Tris-HCl, 6 M urea, 30% [v/v] glycerol, 5% [w/v] SDS) supplemented with 2% (w/v) DTT for 15 min, followed by incubation for 15 min in the same buffer supplemented with 2.5% (w/v) of iodoacetamide. IPG strips were briefly rinsed with SDS running buffer (25 mM Tris, 0.1% [w/v] SDS, 0.002 % [w/v] bromophenol blue) and placed onto 12% acrylamide gels. Strips were then overlaid with 1% (w/v) agarose in SDS running buffer. The second-dimension SDS-PAGE was performed in a SE 600 Ruby unit (GE Healthcare, PA) until the bromophenol blue migrated off the gel. Following SDS-PAGE, gels were stained with Coomassie Colloidal (20% [v/v] ethanol, 1.6% [v/v] phosphoric acid, 8% [w/v] ammonium sulfate, 0.08% [w/v] Coomassie Brilliant Blue G-250) for 24 h. Then Coomasie solution was removed and gels were placed in distaining solution (10 % [v/v] ethanol, 2% [v/v] orthophosphoric acid) twice for 10 minutes, and then placed in a fresh distaining solution overnight. After this time, distaining solution was removed and gels were rinsed twice with deionized water.

*Protein identification*

Two-dimensional gels corresponding to three independent (different biological samples) trials were scanned using a HP M1120 MFP scanner (300 dpi; 16-bit grayscale pixel depth). Fifteen scanned images were analyzed with ImageMaster 2D Platinum software version 7.0 (GE Healthcare, PA) to detect, quantify, and match spots. To compensate for subtle differences in sample loading, gel staining, and fading, the volume of each spot (i.e., spot abundance) was expressed as the relative volume. These gels corresponded to 3 gels per treatment (control, insulin, rapamycin, insulin plus rapamycin, and wound) from 3 independent experiments

each. Proteins were considered differentially expressed at $p < 0.05$, according to the median value obtained using the software.

*In Gel Digestion*

2D gel spots were placed in 1.5 mL eppendorf tubes with 1 mL of water for 30 min. The water was removed and 50 µl of 250 mM ammonium bicarbonate was added. For reduction 5 µL of a 45 mM solution of 1,4-dithiothreitol (DTT) was added and the samples were incubated at 50ºC for 30 min. The samples were cooled to room temperature and then for alkylation, 5 µL of a 100 mM iodoacetamide solution was added and allowed to react for 30 min. The gel spots were washed twice with 1 mL water aliquots. The water was removed and 1mL of 50:50 (50mM Ammonium Bicarbonate:Acetonitrile) was placed in each tube and samples were incubated at room temperature for 1 h. The solution was then removed and 200 µL of acetonitrile was added to each tube at which point the gels slices turned opaque white. The acetonitrile was removed and gel spots were further dried in a Speed Vac. Gel spots were rehydrated in 50 µL of 2 ng/µL trypsin (Sigma) in 0.01% ProteaseMAX Surfactant (Promega):50mM Ammonium Bicarbonate. Samples were incubated at 37ºC for 21 h. The supernatant of each sample was then removed and placed in a separate 1.5 mL eppendorf tube. Gel spots were further dehydrated with 100 µL of 80:20 (Acetonitrile:1% formic acid). The extract was combined with the supernatants of each sample. The samples were then dried down in a Speed Vac. Samples were dissolved in 16 µL of 5% Acetonitrile in 0.1% trifluroacetic acid prior to injection on LC/MS/MS.

*LC/MS/MS on Q Exactive*

A 4.0 µL aliquot out of 16 µL was directly injected onto a custom packed 2 cm × 100 µm $C_{18}$ Magic 5 µ particle trap column. Peptides were then eluted and sprayed from a custom packed emitter (75 µm × 25 cm $C_{18}$ Magic 3 µm particle) with a linear gradient from 95% solvent A (0.1% formic acid in water) to 35% solvent B (0.1% formic acid in Acetonitrile) in 40 min at a flow rate of 300 nL/min on a Waters Nano Acquity UPLC system. Data-dependent acquisitions were performed on a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA) according to an experiment where full MS scans from 300-1750 m/z were acquired at a resolution of 70,000, followed by 10 MS/MS scans acquired under HCD fragmentation at a resolution of 17,500 with an isolation width of 1.6 Da. Raw data files were processed with Proteome Discoverer (version 1.4) prior to searching with Mascot Server (version 2.4) against the Uniprot Maize database. The search parameters utilized were fully tryptic with 2 missed cleavages, parent mass tolerances of 10 ppm, and fragment mass tolerances of 0.05 Da. Variable modifications of acetyl (protein N-term), pyro glutamic for N-term glutamine, oxidation of methionine, and propionamide for cysteine were considered. Search results were loaded into the Scaffold Viewer (Proteome Software, Inc. Portland, OR) to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability by the Peptide Prophet algorithm [15] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at a greater than 90.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the

Protein Prophet algorithm [16]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

## Results

*Insulin induces changes in protein expression during maize germination*

Proteins from embryonic maize axes were resolved by 2D electrophoresis to identify and analyze the differentially expressed proteins. Eighteen spots corresponding to proteins that were differentially expressed were identified when the controls and insulin-treated embryonic axes were compared (Figure 1). Each identified and sequenced spot contained more than one protein, but only the results of the analysis of proteins with the highest score according to the Scaffold software have been presented. A total of 8 proteins were up-regulated, which include proteins involved in processes such as protein folding (Protein Disulfide Isomerase, PDI), regulation of protein degradation (proteosome β regulation subunit), vesicle traffic (Rab-28), desiccation tolerance during embryogenic development (late embryogenesis protein 34, late embryogenesis protein), and glucose metabolism (Enolase-1, Glyceraldehyde 3-phosphate dehydrogenase) (Table 1) [17-19]. On the other hand, we identified 10 down-regulated proteins involved in processes like protein storage and folding (Hsp70, chaperonin CPN60-1, PDI), protection against redox and desiccation stresses (peroxiredoxin-5),

calcium signaling ($Ca^{2+}$ binding protein), and ribonuclease activity (ribonuclease activity regulatory protein) (Table 1) [17, 20-22].

*Stress response presents patterns of protein expression different from those observed during insulin treatment*

Several proteins identified to be differentially expressed during insulin treatment have been related to stress responses, which could indicate that a stress was induced during the manipulation of the embryonic axes. To test this possibility, we cut the axes longitudinally in order to compare the profile of the proteins whose expression was induced by wound stress. The results show that wound stress altered protein expression, as expected, but not in the same way as the expression of proteins modulated by insulin treatment (Table 2). We also tested the effects of other kinds of stress such as heat, dehydration, and osmotic stress; however, none of these induced a protein variation similar to insulin (data not shown).

*Effect of insulin on protein expression is not altered by rapamycin*

Since TOR signaling is involved in the regulation of protein synthesis during plant growth induced by insulin [3, 4, 8], it was evaluated whether this pathway was involved in the insulin-mediated modulation of protein expression. Proteins extracted from the embryonic axes treated with the TOR inhibitor rapamycin were analyzed as described above (Table 3). According to the relative volume of each spot, no differences were observed between the rapamycin treatment group and the insulin-treated group. However, rapamycin inhibits S6K phosphorylation (supplementary figure), thus proving that the macrolide has inhibitory activity.

## Discussion

The isolation of insulin like peptide from maize (ZmIGF) has been previously reported [4, 10]. However, low yield of this peptide during the isolation and purification procedure makes difficult to use it for experiments. On the other hand, human insulin induces similar effects to ZmIGF, promoting germination and plant growth; this is in accordance with modulation of a conserved TOR signaling pathway [3-5, 8]. A putative TOR signal transduction in maize has been proposed previously where Insulin induces activation of TOR, S6K phosphorylation, and accelerates specific protein synthesis, mainly ribosomal proteins [8, 11]. However, the exact mechanisms of the effects of insulin remain elusive. In this work, we aimed to understand how insulin works in plant cells by using a proteomic approach. We identified 18 proteins that are differentially regulated by insulin during maize germination (Figure1, Table 1). We found 8 up-regulated proteins from which, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase are classical enzymes of glycolysis, a common metabolic pathway in eukaryotes and prokaryotes, for the oxidization of hexoses to generate ATP, reducing power, pyruvate, and to produce precursors for anabolism. Over expression of GAPDH in rice, induce high germination rates in response to stress [23], and in the case of enolase in response to gibberellic acid [24]. Late embryogenesis proteins have an important role during germinations as protectors of protein structure, and maintaining homeostasis of membranes during water uptake; over expression of these proteins in response to abscisic acid enhance germination rates in response to drought stress [25]. Rab28 protein is a member of a conserved family of proteins

that regulate protein traffic and is considered as a late embryogenesis protein in plants regulated in response to ABA [26, 27]. PDIL1-1 protein is a member of luminal chaperones proteins that regulates storage proteins folding and traffic like globulins in rice seeds, with a probable role in germination [28]. The function of proteasome complex is degradation of proteins marked with ubiquitin, β subunits have a role in proteasome assembly and conform the proteolityc chamber of it [29]. In plants, proteasome 26s complex is regulated by hormone signaling including auxins, gibberellic acid and abscisic acid, having an important role on growth and development in the germination and life spam of plants [30]. Additionally, 10 down-regulated proteins were identified: HSP70 and CPN60-1 are chaperones members of heat shock proteins family that have roles in protein folding and assembly during germination and abiotic stress conditions [31]. Peroxiredoxin proteins protect the cell from redox stress during water uptake in early stages of germination process [32]. Some of these proteins, such as storage proteins (vicilin and globulins), have been reported to be down-regulated during germination for material recycling [33]. These results indicate that the accelerated germination and plant growth promotion induced by insulin is through mimicking or regulating germination phytohormones, metabolic activation, and storage nutrient degradation. On the other hand, proteins for protection against redox stress, like peroxiredoxin-5, have been reported to be up-regulated during germination in response to plant hormones such as abscisic acid and gibberellic acid [34]. According to the expression ratio analysis from proteins regulated by wound stress, it was confirmed that the expression of stress-related proteins was indeed modulated by insulin treatment and not by manipulation of the embryonic axes (Table 2). Since the TOR signaling pathway is

well known for inducing protein synthesis, we tested whether this pathway is involved in protein expression regulation in response to insulin. Rapamycin, a well-known TOR inhibitor, was used, and the expression ratios were compared to those of the proteins extracted from embryonic axes treated with insulin alone, insulin plus rapamycin, or rapamycin alone (Table 3). Surprisingly, no statistical differences between the insulin and insulin plus rapamycin treatments were observed, indicating that the protein expression modulated by insulin during maize germination is independent of TOR kinase pathways. At this respect, it has been previously reported that the length of Arabidopsis hypocotils was not affected by variations in AtTOR expression, suggesting that the AtTOR kinase does not control cell elongation, at least in etiolated hypocotyls [35]. Taken together, our results show that insulin regulates processes that are directed to promote maize germination. Of special interest are those proteins involved in glucose metabolism, since glucose has been recently reported as a regulator of plant TOR signaling pathways [6] and because of the well-known action of insulin in glucose metabolism.

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## Figure legends

**Figure 1.** Total proteins extracted from embryonic axes resolved by 2D electrophoresis. A total of 300 µg of proteins from control (A) and insulin (1.23 nM) (B) treated axes. The gels were stained with colloidal Coomassie Blue G-250. Arrowheads indicate the spots corresponding to differentially expressed proteins. Represented images from three independent experiments.

**Table 1.** Up and Down-regulated proteins in response to insulin.

| Protein ID | Spot # | Accession | Expression Ratio | % Protein Coverage | No. Unique Peptides | Molecular Weight |
|---|---|---|---|---|---|---|
| Up-regulated Proteins | | | | | | |
| Protein Disulfide Isomerase | 2 | Q5EUE1_MAIZE | 1.39 | 57 | 5 | 57kDa |
| Enolase 1 | 6 | ENO1_MAIZE | 1.45 | 83 | 51 | 48kDa |
| Rab-28 protein | 10 | Q41850_MAIZE | 1.26 | 49 | 11 | 28kDa |
| General Stress Protein 39 | 11 | B4FNZ9_MAIZE | 1.32 | 73 | 45 | 33kDa |
| Glyceraldehyde 3-phosphate dehydrogenase | 12 | B4FAK9_MAIZE | 1.34 | 50 | 3 | 37kDa |
| Proteasome Subunit β type | 14 | B6SIM7_MAIZE | 1.82 | 73 | 9 | 23kDa |
| Late Embryogenesis Protein D-34 | 16 | B6SNS4_MAIZE | 1.95 | 53 | 6 | 21kDa |
| Late Embryogenesis Protein | 18 | B6UH99_MAIZE | 1.51 | 63 | 9 | 16kDa |
| Down-Regulated Proteins | | | | | | |
| Heat Shock Protein 1 (HSP1) | 1 | C4J410_MAIZE | 0.36 | 58 | 14 | 71kDa |
| Protein Disulfide Isomerase | 3 | A5A5E7_MAIZE | 0.09 | 52 | 2 | 57kDa |
| Chaperonin CPN60-1 | 5 | CH61_MAIZE | 0.53 | 62 | 7 | 61kDa |
| Globulin-2 | 8 | Q7M1Z8_MAIZE | 0.42 | 50 | 3 | 48kDa |

| | | | | | | |
|---|---|---|---|---|---|---|
| Globulin-1 | 9 | B6UGJ0_MAIZE | 0.72 | 48 | 3 | 50kDa |
| Calcium Binding Protein | 4 | Q43712_MAIZE | 0.21 | 58 | 10 | 48kDa |
| Vicilin-Like Storage Protein | 7 | Q03865_MAIZE | 0.13 | 25 | 4 | 66kDa |
| Dehydrin-1 | 15 | A3KLI1_MAIZE | 0.79 | 64 | 6 | 17kDa |
| Peroxiredoxin-5 | 17 | B4FN24_MAIZE | 0.36 | 65 | 11 | 17kDa |
| Ribonuclease Activity Regulator Protein | 19 | B6TN41_MAIZE | 0.35 | 48 | 7 | 18kDa |

MASCOT search algorithms from the database for assignation were used, from which assigned peptides with more than 95% of confidence were taken as a correct result.

Accession number from Uniprot Maize database.

Expression ratio (Insulin/control) was determined according to average of relative volume of each spot from three independent experiments.

Percentage of protein coverage from MASCOT search algorithms.

No. of unique peptide represents indicators from different groups of protein.

**Table 2.** Protein expression changes in wound stress compared with insulin.

| Protein ID | Spot # | Accession | Expression Ratio I/C | Expression Ratio W/I | Expression Ratio W/C |
|---|---|---|---|---|---|
| | | Up-regulated | | | |
| Protein Disulfide Isomerase | 2 | Q5EUE1_MAIZE | 1.39 | 1.03 | 1.30 |
| Enolase 1 | 6 | ENO1_MAIZE | 1.45 | 0.67 | 0.83 |
| Rab-28 Protein | 10 | Q41850_MAIZE | 1.26 | 0.94 | 1.13 |
| General Stress Protein 39 | 11 | B4FNZ9_MAIZE | 1.32 | 0.88 | 0.99 |
| Glyceraldehyde 3-phosphate dehydrogenase | 12 | B4FAK9_MAIZE | 1.34 | 0.73 | 0.89 |
| Proteosome Subunit β type | 14 | B6SIM7_MAIZE | 1.82 | 0.97 | 1.69 |
| Late Embryogenesis Protein D-34 | 16 | B6SNS4_MAIZE | 1.95 | 0.58 | 0.80 |
| Late Embryogenesis Protein | 18 | B6UH99_MAIZE | 1.51 | 0.65 | 0.99 |
| | | Down-regulated | | | |
| Heat Shock Protein 1 (HSP1) | 1 | C4J410_MAIZE | 0.36 | 1.23 | 0.42 |
| Protein Disulfide Isomerase | 3 | A5A5E7_MAIZE | 0.09 | 1.28 | 0.16 |
| Chaperonin CPN60-1 | 5 | CH61_MAIZE | 0.53 | 1.15 | 0.66 |
| Globulin-2 | 8 | Q7M1Z8_MAIZE | 0.42 | 0.97 | 0.47 |
| Globulin-1 | 9 | B6UGJ0_MAIZE | 0.72 | 0.94 | 0.63 |
| Calcium binding protein | 4 | Q43712_MAIZE | 0.21 | 1.02 | 0.29 |

| | | | | | |
|---|---|---|---|---|---|
| Vicilin-Like Storage Protein | 7 | Q03865_MAIZE | 0.13 | 0.51 | 0.08 |
| Dehydrin-1 | 15 | A3KLI1_MAIZE | 0.79 | 0.43 | 0.29 |
| Peroxiredoxin-5 | 17 | B4FN24_MAIZE | 0.36 | 1.16 | 0.48 |
| Ribonuclease Activity Regulator Protein | 19 | B6TN41_MAIZE | 0.35 | 0.94 | 0.37 |

Accession number from Uniprot Maize database

Expression ratios: Insulin/ Control (I/C), Wound/Insulin (W/I) and Wound/Control (W/C), were determined according to means of values from the relative volume of each spot from three independent experiments.

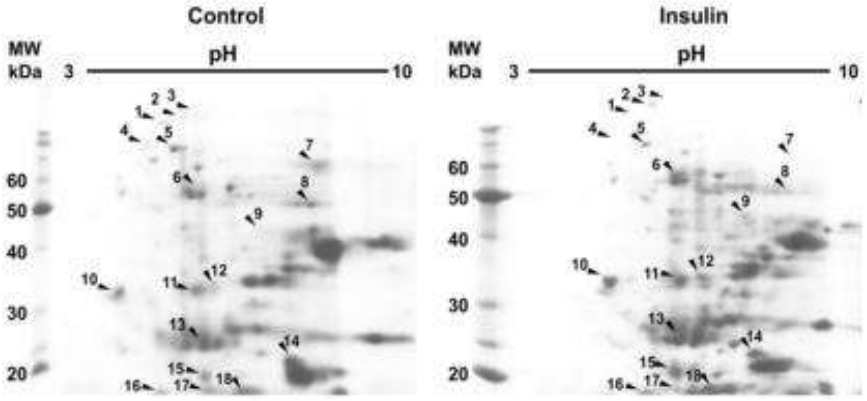
**Table 3.** Protein expression changes induced by rapamycin.

| Protein ID | Spot # | Accession | Expression Ratio I/C | Expression Ratio R/I | Expression Ratio R/C | Expression Ratio I+R/C | Expression Ratio I+R/I |
|---|---|---|---|---|---|---|---|
| | | | Up-regulated | | | | |
| Protein Disulfide Isomerase | 2 | Q5EUE1_MAIZE | 1.39 | 0.97 | 1.28 | 1.35 | 1.02 |
| Enolase 1 | 6 | ENO1_MAIZE | 1.45 | 0.95 | 1.18 | 1.09 | 0.97 |
| Rab-28 Protein | 10 | Q41850_MAIZE | 1.26 | 0.77 | 0.98 | 1.03 | 0.83 |
| General Stress Protein 39 | 11 | B4FNZ9_MAIZE | 1.32 | 0.88 | 1.06 | 1.03 | 0.90 |
| Glyceraldehyde 3-phosphate dehydrogenase | 12 | B4FAK9_MAIZE | 1.34 | 0.69 | 0.78 | 0.83 | 0.76 |
| Proteosome Subunit β type | 14 | B6SIM7_MAIZE | 1.82 | 0.75 | 1.06 | 0.98 | 0.81 |
| Late Embryogenesis Protein D-34 | 16 | B6SNS4_MAIZE | 1.95 | 0.62 | 1.03 | 1.06 | 0.58 |
| Late Embryogenesis Protein | 18 | B6UH99_MAIZE | 1.51 | 0.70 | 1.03 | 1.08 | 0.77 |
| | | | Down-regulated | | | | |
| Heat Shock Protein 1 (HSP1) | 1 | C4J410_MAIZE | 0.36 | 2.7 | 1.18 | 1.16 | 0.87 |
| Protein Disulfide Isomerase | 3 | A5A5E7_MAIZE | 0.09 | 1.12 | 0.07 | 0.09 | 1.08 |
| Chaperonin CPN60-1 | 5 | CH61_MAIZE | 0.53 | 1.54 | 1.02 | 0.97 | 1.46 |
| Globulin-2 | 8 | Q7M1Z8_MAIZE | 0.42 | 1.57 | 0.96 | 1.02 | 1.60 |
| Globulin-1 | 9 | B6UGJ0_MAIZE | 0.72 | 1.30 | 0.94 | 0.99 | 1.23 |
| Calcium binding protein | 4 | Q43712_MAIZE | 0.21 | 1.74 | 0.91 | 1.02 | 1.71 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Vicilin-Like Storage Protein | 7 | Q03865_MAIZE | 0.13 | 7.35 | 0.95 | 0.97 | 7.32 |
| Dehydrin-1 | 15 | A3KLI1_MAIZE | 0.79 | 1.14 | 0.90 | 0.93 | 1.16 |
| Peroxiredoxin-5 | 17 | B4FN24_MAIZE | 0.36 | 2.92 | 1.05 | 0.99 | 2.89 |
| Ribonuclease Activity Regulator Protein | 19 | B6TN41_MAIZE | 0.35 | 3.14 | 1.10 | 1.07 | 3.18 |

Accession number from Uniprot Maize database.

Expression ratios: Insulin/Control (I/C), Rapamycin/Insulin (R/I), Rapamycin/ Control (R/C), Insulin+Rapamycin/Control (I+R/C), Insulin+Rapamycin/Insulin (I+R/Insulin), were determined according to means of values from the relative volume of each spot from three independent experiments.



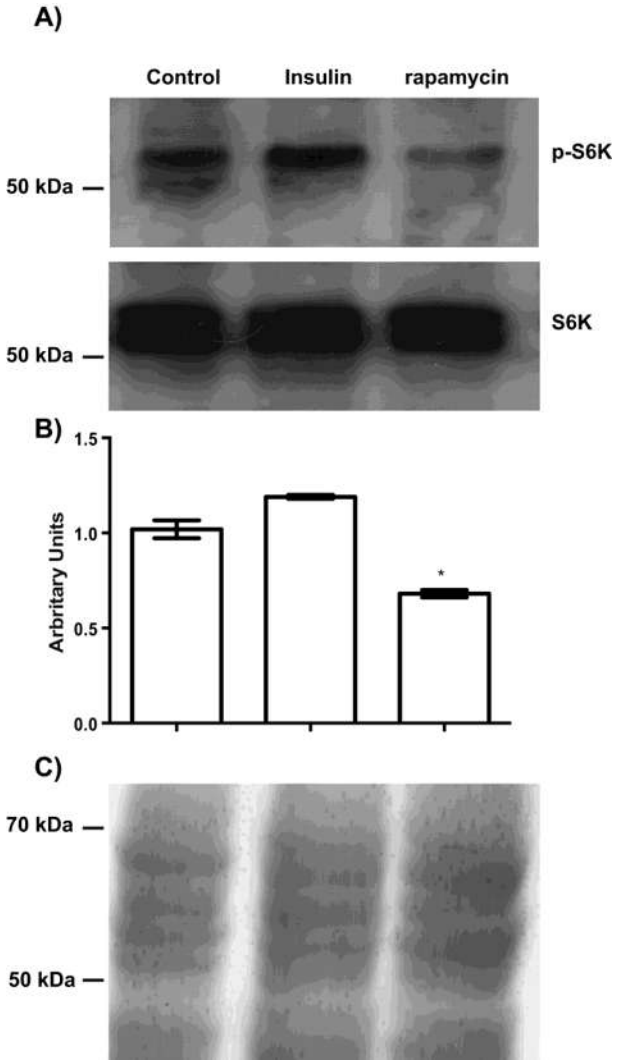

75x33mm (600 x 600 DPI)

**Supporting Information**

**Supplementary Figure.** Effect of insulin and rapamycin on S6K phosphorylation. A) Twenty µg of proteins from embryonic axes, control and treated with insulin (1.23 nM) or rapamycin (100 nM) were analyzed by Western blot using anti p-S6K antibodies (upper panel) and anti S6K protein (lower panel). B) Densitometric analysis of Western blots. C) Coomassie stained gel was used to confirm protein amounts loaded per lane. Images are representative of at least 3 independent experiments. Densitometric analysis are means ± standard error, one-way analysis of variance was performed $p>0.05$.

**Supplementary methodology**

Maize embryonic axes were treated as described in material and methods with Insulin (1.23 nM) or rapamycin (100 nM). Then the axes were rinsed with deionized water, frozen with liquid nitrogen, ground in a mortar, and homogenized in freshly prepared extraction buffer [50 mM HEPES (pH 7.6), 50 mM $Na_4P_2O_7$, 1 mM $Na_2VO_4$, 1 mM $Na_2MoO_4$, 4 mM EDTA, 20 mM EGTA, 20 mM NaF, 80 mM □-glycerophosphate, 200 mM mannitol, 2 mM DTT, 0.2 mM PMSF, and 1 mM benzamidine]. The homogenate was centrifuged at 9,000*g* for 30 min at 4C, supernatant (protein extract) was separated and analyzed immediately or stored at -80 ºC. Protein quantification was performed by Bradford method [12]. Equal amounts of axes extract (20µg of protein) were resolved in 12% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were assayed by Western blot using anti phospho-human p70S6K-Thr389 (p-S6K), or anti human p70S6K1 (S6K) at 1:2000 dilution (Santa Cruz Biotechnology). Prism 5 (Graphpad software, La Jolla, CA) was used for statistical analyses of densitometric values obtained with Image-J software (ImageJ 1.48v NIH, USA). Data were expressed as means ± SE and were analyzed by one-way ANOVA analysis. Differences were considered significant at $p<0.05$.


A)
Control
Insulin
rapamycin
50 kDa
p-S6K
50 kDa
S6K
B)
Arbritary Units
1.5
1.0
0.5
0.0
*
C)
70 kDa
50 kDa

## VII. Discusión general.

En mamíferos es común encontrar hormonas de naturaleza peptídica que son capaces de inducir respuestas celulares por medio de diversas rutas de señalización, un ejemplo de esto es la insulina, y los péptidos similares a esta que funcionan como factores de crecimiento. Actualmente sabemos que este tipo de péptidos se encuentran no solo en vertebrados, sino que se han encontrado en una gran variedad de organismos como insectos y moluscos (Ebberink et al, 1989). En plantas recientemente también se han reportado péptidos encargados de inducir señalización para producir una gran variedad de respuestas celulares (Katsir et al, 2011); algunos ejemplos son: el péptido fitosulfocin-alfa (PSK) que regula la proliferación celular (Matsubayashi & Sakagami, 1996); el gen ENOD40 codifica para dos péptidos con función de regulación del uso de la sacarosa sintasa (Rohrig et al, 2002); CLAVATA3 codifica para un péptido encargado de regular procesos de proliferación y diferenciación, principalmente en el meristemo (Fiers et al, 2007). Dentro de los péptidos conocidos en plantas, las defensinas fueron las primeras en ser descritas y han sido las de mayor objeto de estudio. Estos péptidos son capaces de inhibir el crecimiento de hongos y bacterias patógenas de las plantas, por lo que están involucradas en mecanismos de defensa (Broekaert et al, 1995). A partir de estos descubrimientos se ha hecho evidente que estos péptidos juegan un papel importante en funciones de señalización que regulan procesos como el crecimiento, desarrollo vegetal y mecanismos de defensa.

El péptido ZmIGF tiene una secuencia anotada en el GenBank con el número P81009 que está relacionada a un reporte de dos secuencias de dos γ-tioninas de semillas de maíz (Castro et al, 1996). Tioninas fue el nombre con el que se les conocía anteriormente a las defensinas, es decir, el ZmIGF es una defensina en cuanto a secuencia de aminoácidos se refiere. Sin embargo Garrocho y col. Indicaron que éste péptido presenta similitud estructural en la cadena B de la insulina así como a factores de crecimiento tipo insulina de mamíferos y además es capaz de inducir la toma de glucosa en adipocitos de rata.

La obtención y purificación del ZmIGF resulta en un proceso costoso con bajo rendimiento (Rodríguez-lópez et al., 2011), por lo que es poco práctico usarlo para estudiar la respuesta que desencadena. La insulina animal ha sido usada para estudiar esta respuesta en plantas (Sánchez de Jiménez et al., 1999; García Flores et al, 2001; Dinkova et al., 2007).

En este trabajo hemos usado insulina humana como tratamiento para ejes embrionarios de maíz de 24 horas de germinación, ya que de acuerdo a lo reportado por García-Flores y col., es durante la germinación cuando el ZmIGF se expresa, alcanzando una mayor nivel a las 48 horas de germinación. El nivel de expresión a las 24 horas nos permite agregar la insulina extra para analizar la respuesta a esta. En los trabajos mencionados anteriormente, únicamente se hace relación a la vía de señalización PI3K-TOR indicando que se modula la síntesis de proteínas ribosomales. En este trabajó se realizó un análisis del proteoma de los ejes embrionarios de maíz tratados con la insulina como medio para indagar en la respuesta a ésta desde un enfoque diferente. Los resultados nos muestran una amplia gama de funciones celulares involucradas de las 18 proteínas identificadas, las reguladas en un aumento en su expresión están involucradas en: Metabolismo de carbohidratos, protección de estructura proteica, mantenimiento de la homeostasis durante la toma de agua en la germinación, degradación de proteínas ubiquitinadas; todas ellas importantes para llevar una germinación exitosa. Dentro de las que mostraron una disminución en su expresión están funciones como: plegamiento de proteínas, degradación de proteínas de almacenaje y protección contra estrés producido por generación de radicales libres; al igual que las anteriores, importantes durante la germinación. Estos resultados indican que la inducción de la germinación por parte de la insulina desencadena procesos similares a los inducidos por reguladores del crecimiento vegetal que promueven la germinación, como el ácido abscísico y las giberelinas (Hilhorst & Karssen, 1992); para llevar a cabo una germinación exitosa en maíz.

Por otro lado, el uso de la Rapamicina se planteó para explorar si estas modificaciones en los patrones de expresión se debían a la acción de la vía de

señalización de TOR. Los resultados nos mostraron que la Rapamicina al inhibir a TOR no modificó el patrón de expresión de las proteínas moduladas por la insulina. Esto nos indica que los posibles mecanismos involucrados son independientes de TOR, por lo que sería interesante explorar la posibilidad de respuestas involucrando reguladores del crecimiento vegetal como el ácido abscísico o las giberelinas.

Como se menciono al principio de este apartado, la secuencia del ZmIGF lo ubica como una defensina, proteínas que se expresan en respuesta un ataque por patógenos. Sin embargo, éste se aumenta su expresión durante la germinación del maíz, mostrando funciones diferentes a las defensinas comunes. Un ejemplo es la inducción de síntesis de proteínas ribosomales mediante la activación de la vía de la proteína TOR. En este trabajo hemos reportado que además de esas funciones, induce una serie de eventos dirigidos a mantener una germinación exitosa en el maíz. Dadas las características presentadas por Garrocho-Villegas y col. sería de gran interés averiguar a nivel evolutivo las características estructurales y funcionales de la insulina en diferentes organismos incluidas las plantas.

## VIII. Conclusión general.

De acuerdo a lo observado en este trabajo podemos concluir que la insulina induce cambios en la expresión de proteínas involucradas en diferentes procesos celulares, dirigidos principalmente a conducir exitosamente la germinación de la planta. Además, estos procesos parecen ser independientes de la vía de señalización de la proteína TOR, que es el principal referente en el que se ha visto involucrada la respuesta de esta hormona en plantas. Dada la similitud que hay entre esta respuesta observada a insulina y los procesos que son regulados por fitohormonas como el ácido abscísico y las giberelinas, sería interesante explorar una posible conexión entre ellas como un mecanismo alterno al llevado a cabo por la vía de señalización de la proteína TOR.

# XIX Anexo

**Publicaciones alternas.**

Plant Growth Regul
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ORIGINAL PAPER

# Oligogalacturonides inhibit growth and induce changes in S6K phosphorylation in maize (*Zea mays* L. var. Chalqueño)

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**Abstract** Cell growth is regulated by the target of rapamycin (TOR) signaling pathway, which integrates environmental cues in eukaryotes. In plants the final organ size is determined by the number and cell size. Several proteins involved in the TOR signaling pathway are conserved in plants, although a differential regulation has been proposed because insensitivity to rapamycin by *Arabidopsis thaliana* was reported. Reports about the role of auxin in the activation of maize S6 ribosomal protein kinase (S6K), a downstream substrate of TOR, have been published indicating a central role of the TOR pathway in the regulation of plant growth. However, in addition to phytohormones, there are a variety of plant growth regulators, including cell wall fragments called oligogalacturonides (OGs). Here we report the effect of OGs on maize growth and development, as well as on S6K activation. We found that oligogalacturonides inhibit coleoptile growth and modify root architecture of maize seedlings. Western blot analyses indicated a modulation of maize S6K activity from seedlings and embryonic axes in response to OGs treatment. These results show that oligosaccharides regulate growth and development through the modulation of TOR signaling pathway in maize.

**Keywords** Oligogalacturonides · Seedling growth · TOR signaling · Plant growth and development

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**Abbreviations**

| | |
|---|---|
| OGs | Oligogalacturonides |
| TOR | Target of rapamycin |
| S6rp | S6 ribosomal protein |
| S6K | S6rp kinase |
| eIF4E | Eukaryotic translation initiation factor 4E |
| 4EBP1 | eIF4E binding protein |
| TORC | TOR complex |
| FKBP12 | FK506-binding protein |
| IGF | Insulin-like growth factor |
| IAA | Indole-3-acetic acid |

## Introduction

Plant growth mainly depends on increase in cell number or proliferation and cell size or cell growth. Cell proliferation occurs in plant meristems, zones with high mitotic activity where total number of cells is produced, and which ultimately are responsible for plant growth and development. When plant cells leave the meristematic zone, they succumb to several processes that lead to cell growth and differentiation (development). Cell growth in eukaryotes results from biomass increase, mainly due to greater protein synthesis, including ribosome biogenesis (Jastrzebski et al. 2007). These last events involve great amount of cell energy, so their regulation must be closely related to cell nutritional state. The TOR (target of rapamycin) kinase integrates stimuli from growth factors, mitogens, and nutrients to regulate ribosome biogenesis and protein synthesis rates, through downstream protein target activation, and has been proposed as central cell growth controller in non plant eukaryotes (Wullschleger et al. 2006). The main TOR protein targets are the S6 ribosomal protein (S6rp) kinases (S6Ks), and the eukaryotic

translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1; Burnett et al. 1998). Rapamycin binds to FKBP12 (FK506-binding protein) to form a complex that in turn binds TOR, thereby inhibiting signaling to S6K1 and 4EBP1 (Avruch et al. 2006). TOR forms two functional complexes, a rapamycin-sensitive TOR complex 1 (TORC1), which includes the scaffolding protein raptor, and a rapamycin-insensitive complex 2 (TORC2) that contains rictor (Loewith et al. 2002; Yang and Guan 2007). TORC1 activates p70S6K, which promotes translation by phosphorylating the S6 ribosomal protein and phosphorylating 4EBP1, allowing the release of eIF4E and further promoting translation of Cap-dependent transcripts (Hara et al. 2002).

On the other hand, the knowledge of TOR signaling pathway is scarce in plants; however some components are present in *Arabidopsis thaliana*, and are ideal candidates for operating the link between nutritional sensing and the regulation of growth (Menand et al. 2004). TOR proteins have been reported in Arabidopsis (Menand et al. 2002) and maize (Agredano-Moreno et al. 2007). There are two S6K genes, S6K1 and S6K2 in Arabidopsis, having a highly similar sequence and function (Henriques et al. 2010). Arabidopsis S6K2 is able to carry out conserved signaling functions, because it could be activated by insulin in a TOR-dependent manner, when introduced into human cells (Turck et al. 1998). The growth hormones, auxins and cytokinins enhance S6rp phosphorylation in Arabidopsis cell cultures (Turck et al. 2004), whereas stress factors, such as heat and oxidative stress rapidly block it (Williams et al. 2003). Additionally, there are several reports about the conserved signaling pathway in maize, including the existence of an insulin-like growth factor (IGF; García Flores et al. 2001; Rodriguez-Lopez et al. 2011); and the maize S6K (ZmS6K; Reyes de la Cruz et al. 2004). This last protein is responsible for in vivo S6rp phosphorylation in an insulin dependent manner through TOR signaling pathway and this process was rapamycin sensitive (Reyes de la Cruz et al. 2004).

The control of plant growth and development is orchestrated by plant growth regulators known as phytohormones. Diverse environmental stimuli lead to biosynthesis and perception of such plant hormones. Phytohormones regulate diverse aspects of growth and development in plants including cell elongation which is dependent on cell wall dynamics. The cell wall degradation and biosynthesis is a process that mainly depends on different phytohormones signaling pathways (Sánchez-Rodríguez et al. 2010). Cell wall is composed of different polysaccharides such as cellulose, hemicelluloses and pectin. Pectin is composed of three different 1,4-linked α-D-galactosyluronic acid based polysaccharide chains (homogalacturonan, rhamnogalacturonan I and II; Ridley et al. 2001; Humphrey et al. 2007). It has been established that homogalacturonan fragments called oligogalacturonides (OGs), are related to plant response to pathogen attacks, inducing phytoalexins biosynthesis (Bergmann et al. 1994; Davis and Hahlbrock 1987; Hahn et al. 1981; Hernández-Mata et al. 2010; Nothnagel et al. 1983). On the other hand, these fragments of homogalacturonan had been reported to participate in plant growth and development (Bellincampi et al. 1993; Hernández-Mata et al. 2010; Marfà et al. 1991). Recently, has been reported that OGs modulate the root architecture in *Arabidopsis thaliana*. These OGs induce PAD3 expression and camalexin synthesis, as well as primary root growth inhibition and increased lateral root and root hair formation; these alterations were attributed to altered auxin responses mediated by flavonoids (Hernández-Mata et al. 2010). In addition, an interaction of auxins with galactoglucomannan oligosaccharides (GGMOs) in the regulation of mung been primary root growth has been reported (Kollárová et al. 2010).

Given the relationship between auxins signaling and cell wall dynamics, and that both compounds (auxins and OGs) regulate plant growth and development, and since auxins effects are, at least in part, through TOR signaling pathway, we wondered whether the OGs could modulate plant growth interfering with such pathway. The aim of this work was to analyze the maize S6K activation in response to OGs and compare it with IAA treatments. Data presented herein suggest that in maize OGs treatment inhibited plant growth and modified root architecture, perhaps through modulating S6K phosphorylation. These results suggest that the regulation of plant growth and development by OGs is related to TOR signaling pathway activation.

## Materials and methods

### Biological material

For all experiments, maize embryonic axes (*Zea mays* L. cv. Chalqueño) were used. Seeds were decontaminated using ethanol (70%) for 5 min and placed in sterile water-imbibed cotton beds for germination (24 h, 25 ± 2°C in darkness). In the next step, embryonic axes were manually dissected, decontaminated and placed on Murashige-Skoog medium (MS; Murashige and Skoog 1962) supplemented with plant cell culture tested agar (SIGMA, 7.5 g per liter).

OGs were obtained by enzymatic hydrolysis of *Phaseolus vulgaris* cv. "flor de mayo" cell walls as described in Hernández-Mata et al. (2010). The OGs mixture from linear homogalacturonan fragments with a polymerization

degree of 6–13 D-galacturonic acid residues, was used in all experiments.

### Seedling growth

Basal MS media were prepared [salts according to Murashige and Skoog (1962), glycine 26 mM, *myo*-inositol 55 mM, nicotinic acid 4 mM, pyridoxal 24 mM, thiamine 3 mM, sucrose 87.6 mM, and agar 7.5 g per liter], with addition of different OGs concentrations (0.017, 0.17, 1.7, 17, 170, and 1,700 ng $mL^{-1}$) and indole-3-acetic acid ($1 \times 10^{-7}$, $1 \times 10^{-8}$, $1 \times 10^{-9}$, $1 \times 10^{-10}$, $1 \times 10^{-11}$, and $1 \times 10^{-12}$ M). Rapamycin 100 nM was used because this concentration was reported to inhibit maize S6K (Reyes de la Cruz et al. 2004). Five millilitres of medium containing different substances were placed into 20 × 150 mm tubes in sterile conditions.

Embryonic axes were decontaminated using commercial NaClO (50%) for 10 min, after washing three times with sterile deionized water, they were planted one axe per tube, with the radicle in the medium. Growth conditions were: 25 ± 2°C, darkness for 10 days. We used 10 tubes per treatment with at least three replicates for each experiment. At the end of treatment coleoptile and primary root length, as well as lateral roots number were evaluated.

### Western blots

Ten days old plants were frozen with liquid nitrogen and ground in a mortar, and homogenized in freshly prepared extraction buffer [50 mM HEPES (pH 7.6), 50 mM $Na_4P_2O_7$, 1 mM $Na_2VO_4$, 1 mM $Na_2MoO_4$, 4 mM EDTA, 20 mM EGTA, 20 mM NaF, 80 mM $\beta$-glycerophosphate, 200 mM mannitol, 2 mM DTT, 0.2 mM PMSF, and 1 mM benzamidine]. The homogenate was centrifuged at 9,000*g* for 30 min at 4°C, supernatant (axes extract) was separated and analyzed immediately or stored at −70°C. Protein quantification was performed by Bradford method (Bradford 1976). Equal amounts of axes extract (at least 150 µg of protein) were resolved by two-dimensional electrophoresis according to O'Farrell method (O'Farrell 1975). pH gradient used in the first dimension was in the range of 3.5–10 amplifying the 6–8 range. Second dimension was performed in 12% SDS–polyacrylamide gels. Then the second dimension gels were transferred to PVDF membranes (Millipore) and assayed by Western blot using anti-human p70S6K (Santa Cruz Biotechnology).

Embryonic axes of 24 h germinated seeds were treated with or without OGs 680 ng $mL^{-1}$, Insulin 1.23 nM, IAA 0.4 µM or a OGs-IAA combination during 2 h in liquid MS medium. After this, the embryonic axes were washed with deionized water and axe extracts were prepared as above. Proteins (30 µg) from this extracts were resolved in 12% SDS–polyacrylamide gel, transferred to PVDF membranes and analyzed by Western blot using anti phospho-human p70S6K-Thr389, or anti human p70S6K1 from Santa Cruz Biotechnology.

### Statistical analysis

Prism 5 (Graphpad software, La Jolla, CA) was used for all statistical analyses. Data were expressed as means ± SE and were analyzed by one-way ANOVA followed by Tukey post hoc analysis. Differences were considered significant at $P < 0.05$.

## Results

### OGs modulate S6K phosphorylation of maize embryonic axes

Previously, during maize germination an increase of S6K activation was detected (Reyes de la Cruz et al. 2004). Embryonic axes treatment with insulin 200 µU (1.23 nM) induced S6 ribosomal protein phosphorylation and increase of protein synthesis (Sánchez de Jiménez et al. 1999). In previous work we also show that OGs 680 ng $mL^{-1}$ inhibit coleoptile growth of maize, and that IAA 0.4 µM has an opposite effect on this process (unpublished data). To determine whether OGs modulate the S6K phosphorylation during germination we treated maize embryonic axes of 24 h germinated seeds with the OGs mixture, IAA or both compounds in combination. Additionally, we included a set of embryonic axes treated with Insulin, which has been demonstrated to stimulate S6K phosphorylation (Reyes de la Cruz et al. 2004). Extracts from the axes were prepared and resolved by SDS-PAGE and Western blot using either anti-human p70S6K1 or anti-phospho human p70S6K-Thr389 antibodies (Fig. 1a). Densitometric analysis was performed to the Western blot (Fig. 1b) normalized with the protein loading control observed in the Coomassie stained gel (Fig. 1c). Our results showed that insulin, IAA and OGs increase S6K phosphorylation after 2 h of treatment compared to the control (Fig. 1a, b). Combination of OGs and IAA induced a higher phosphorylation of S6K, suggesting a synergism in the activity of both compounds (Fig. 1a, b). This result indicates that during germination with a short period of OGs treatment (2 h), this last compound modulates S6K phosphorylation in maize embryonic axes.

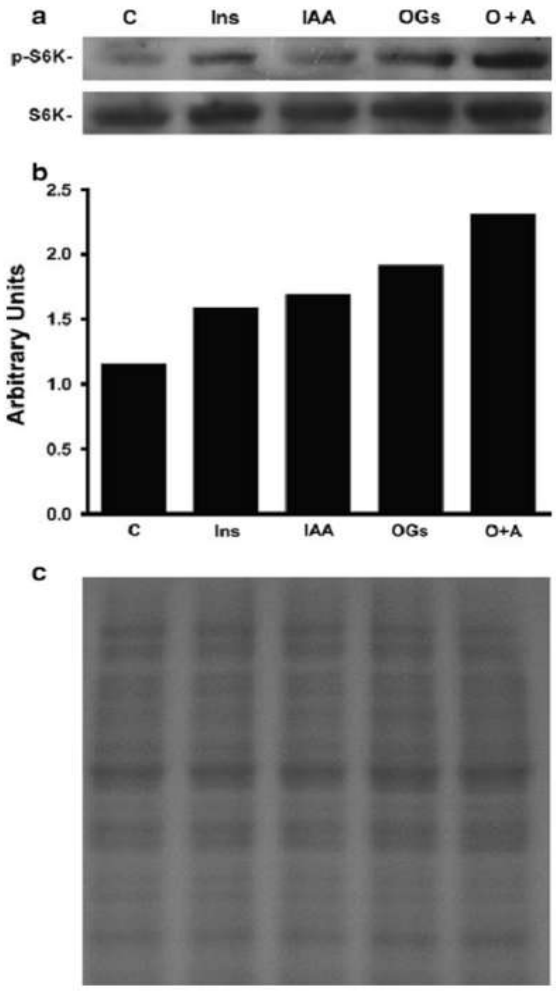


**Fig. 1** OGs modulates S6K phosphorylation in maize embryonic axes. **a** Protein extracts of maize axes from 24 h germinated control seeds (C), or treated with Insulin (Ins), Indole-3-acetic acid (IAA), Oligalacturonides (OGs), or the IAA plus OGs combination (O + A) were resolved (30 µg of protein) by SDS-PAGE and analyzed by Western blot using anti-human p-p70S6k1 (Thr-389) antibody (*upper panel*) and anti-human p70S6K1 antibody (*lower panel*). **b** Densitometric analysis (p-p70S6K/p70S6K) from (**a**) after correction by protein loading. **c** Coomasie stained gel used as load control in (**b**). Results are representative from at least three independent experiments

## OGs modulates maize growth and development

In order to determine whether OGs could affect maize seedling growth or development, we tested different concentrations of OGs mixture. After 10 days of treatment whit OGs, a coleoptile growth inhibition at all concentrations tested was evident; however, this effect was more pronounced at 1,700 ng $mL^{-1}$ of OGs (Fig. 2a, c). These results are in agreement with previous reports where OGs treatment inhibited plant growth (Hernández-Mata et al. 2010); or GGMOs which showed similar effect (Kollárová et al. 2010). OGs treatment modulate root architecture by inhibiting primary root length (Fig. 2b) and by increasing lateral root number (Fig. 2d), as has been also reported by Hernández-Mata et al. (2010) in Arabidopsis.

The OGs effects observed were very similar to those reported by auxins, suggesting that the changes effected by OGs in the root system architecture appear to be mediated at least in part by these phytohormones. For this reason, we tested different concentrations of indole-3-acetic acid (IAA) to compare auxins effects with those induced by OGs. We found the known effects of auxins on plant growth: a slight although not statistically significant increase in coleoptile (Fig. 3a, c) and primary root length (Fig. 3b) at the lower concentrations of IAA ($1 \times 10^{-12}$ M) and coleoptile and primary root length inhibition at the higher concentrations (Fig. 3a, b). A characteristic increase in lateral root number was also observed at $1 \times 10^{-12}$ M of IAA (Fig. 3d). Hence we chose this last IAA concentration to contrast with the OGs effect.

## OGs, IAA and rapamycin effect on maize growth and development

To analyze the participation of the TOR signaling pathway on the effects shown by OGs and IAA, we used OGs 1,700 ng/ml, concentration that inhibits coleoptile growth and induces lateral root formation, and $1 \times 10^{-12}$ M IAA, which does not inhibit coleoptiles growth, but increases lateral root number. On the other hand, we used the TOR complex 1 specific inhibitor rapamycin to analyze the participation of this signaling pathway in OGs and IAA action. Our results shown that the inhibitory effect of OGs on coleoptiles growth was not overcome in the presence of IAA, at least it is not statically significant (Fig. 4a, O + A). Rapamycin treatment inhibits coleoptile and primary root growth as well as lateral root formation after 10 days (Fig. 4). This result indicates, that as previously reported, maize unlike Arabidopsis, is sensitive to rapamycin (García Flores et al. 2001; Reyes de la Cruz et al. 2004). After OGs plus rapamycin treatment, not enhanced inhibition has been observed on coleoptiles growth (Fig. 4a, d, O + R), indicating a possible interaction of OGs with TOR signaling pathway. This is suggested since, if OGs effect was independent on TOR signaling we should observe a potentiated inhibition of coleoptile growth by rapamycin. In contrast, all the measured parameters resulted higher in the combined effect (O + R) compared with R (Fig. 4), but the only inhibitory effect of rapamycin on lateral root formation was statistically overcome by OGs in the combined treatment (Fig. 4c

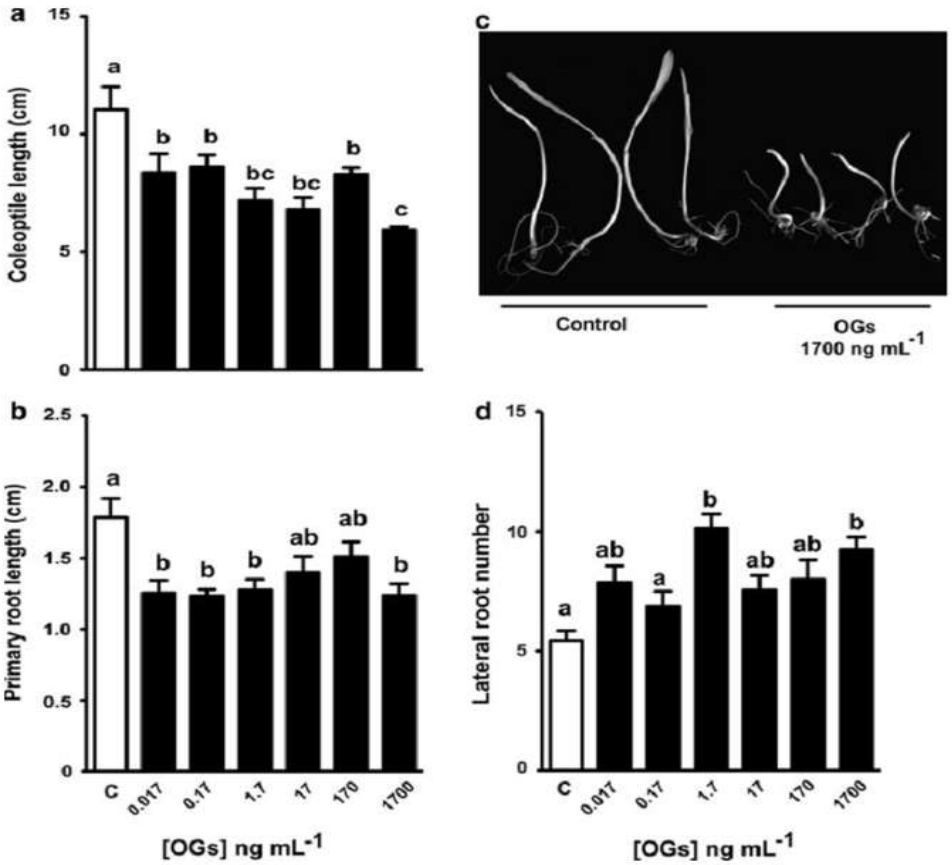


**Fig. 2** OGs effect on maize growth and development. Maize seedlings were grown for 10 days under increasing OGs concentrations and coleoptile length (**a**) primary root length (**b**) and total lateral roots number (**d**) were evaluated. The values represent the mean ± SE from 30 seedlings. *Different letters* represent means statistically different at $P < 0.05$. **c** Representative picture showing maize seedlings—control and with OGs 1,700 ng $mL^{-1}$ in the MS medium

O + R), further indicating an interaction of both compounds at this level.

S6K electrophoretic changes in response to OGs or IAA

To test whether the OGs inhibitory effect is due to an interaction with TOR signaling network, we resolved maize extracts from different treatments in 2D electrophoresis and used commercial antibodies against human p70S6K1 protein [previously reported to recognize maize S6K (Reyes de la Cruz et al. 2004)]. In the 2D electrophoresis, proteins are separated in agreement to their electric charge, phosphorylated proteins are negatively charged and then migrate to the positive pole. Therefore, signals that appear towards positive pole represent hyper-phosphorylated protein forms, whereas signals moving to negative pole represent hypo-phosphorylated or not phosphorylated protein forms. The results show the basal phosphorylation state of maize S6K in the control group (Fig. 5, C). Treatment with rapamycin produces an electrophoretic movement towards negative pole, meaning that rapamycin is blocking TOR signaling to S6K as seen by appearance of hypo-phosphorylated forms of the maize S6K, that suggest an inactivation of this protein (Fig. 5, Rapamycin). Similar effect was detected with OGs indicating that OGs growth inhibitory effect is due to inactivation of TOR signaling pathway (Fig. 5, OGs). Interestingly, IAA shows not only hyper-phosphorylated forms but changes in protein weight can be observed. This indicates possible post-translational modification in addition to phosphorylation that changes the S6K molecular weight and could affect their activity (Fig. 5, IAA). Surprisingly, the combined treatment of OGs with IAA results in the appearance of several spots in both, IEF and molecular weight (Fig. 5, OGs + IAA), which is consistent with the results seen in maize embryonic axes (Fig. 1), where increased S6K phosphorylation was observed. In the OGs + Rap combined treatment the spots observed have a displacement to positive pole, further indicating an interaction of these compounds at the S6K phosphorylation or post translational modification level.

## Discussion

This work provides evidence that OGs effect on growth or plant development, at least in part, is due to modulation of

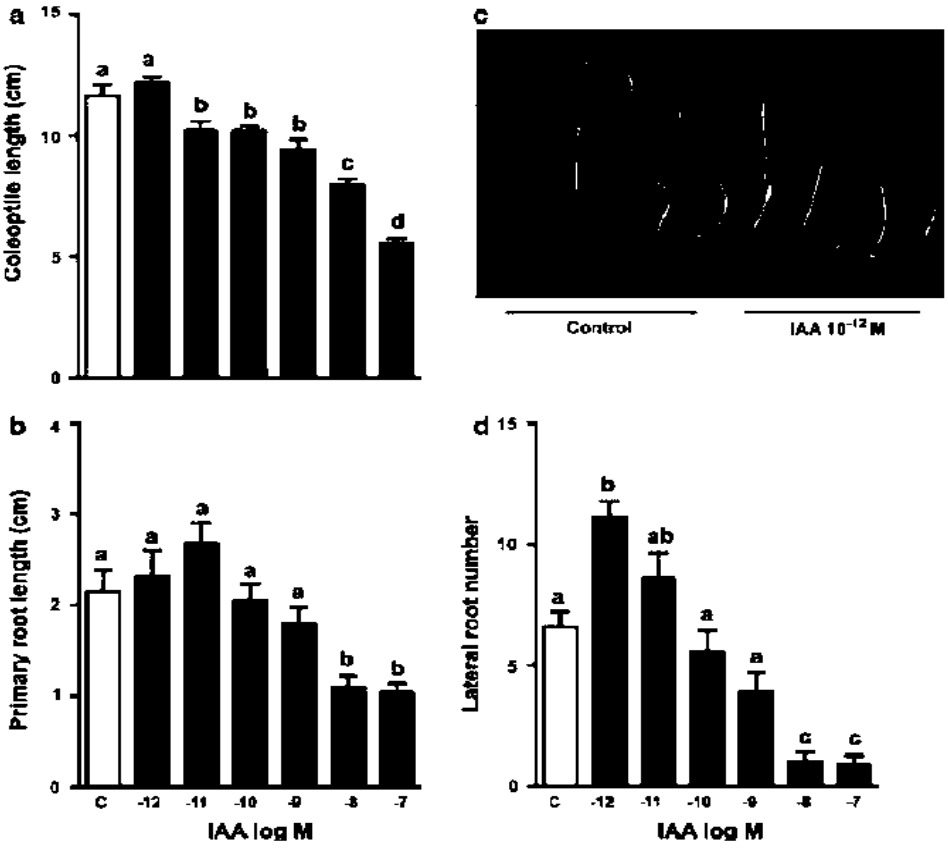


**Fig. 3** IAA effect on maize growth and development. Maize seedlings were grown for 10 days under increasing IAA concentrations and coleoptile length (**a**) primary root length (**b**) and total lateral roots number (**d**) were evaluated. The values represent the mean ± SE from 30 seedlings. *Different letters* represent means statistically different at $P < 0.05$. **c** Representative picture showing maize seedlings—control and with IAA $1 \times 10^{-17}$ M

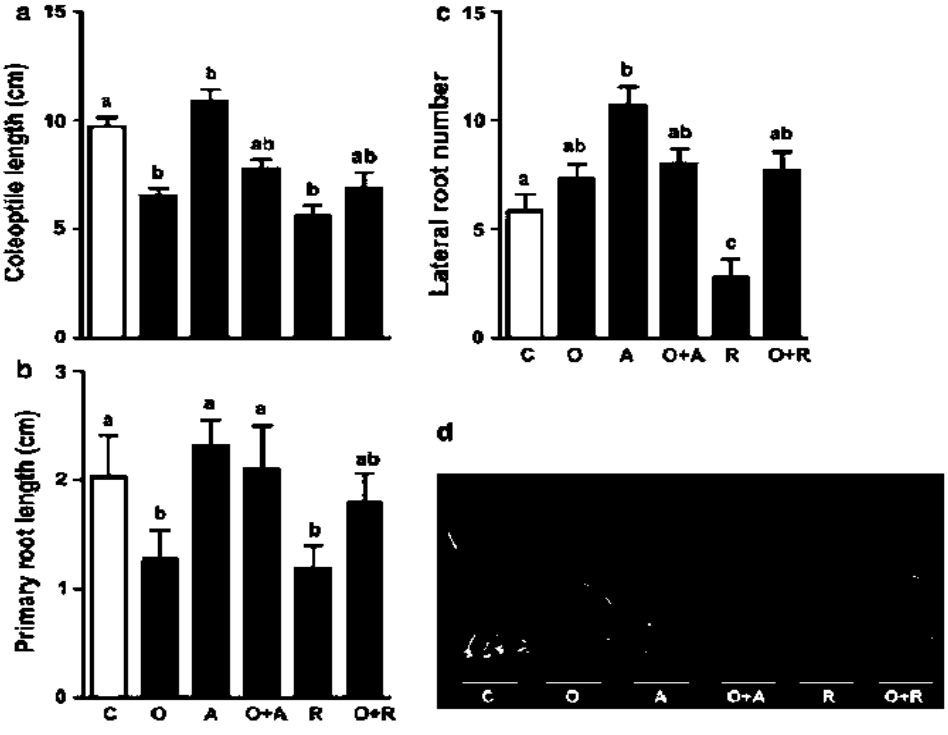


**Fig. 4** Effect of combined treatment on maize growth and development. Maize seedlings were grown for 10 days in the control conditions and with OGs 1,700 ng $mL^{-1}$ (O), IAA $1 \times 10^{-12}$ M (A), Rapamycin 100 nM (R), and the combination of these compounds in MS medium: OGs plus IAA (O + A); OGs plus Rapamycin (O + R). All samples were compared with the control. After this period, the coleoptile (**a**) and primary root length (**b**), as well as total lateral roots number (**c**) was evaluated. The values represent the mean ± SE from 30 seedlings. *Different letters* represent means statistically different at $P < 0.05$. **d** The photograph represents plants from one of three independent experiments

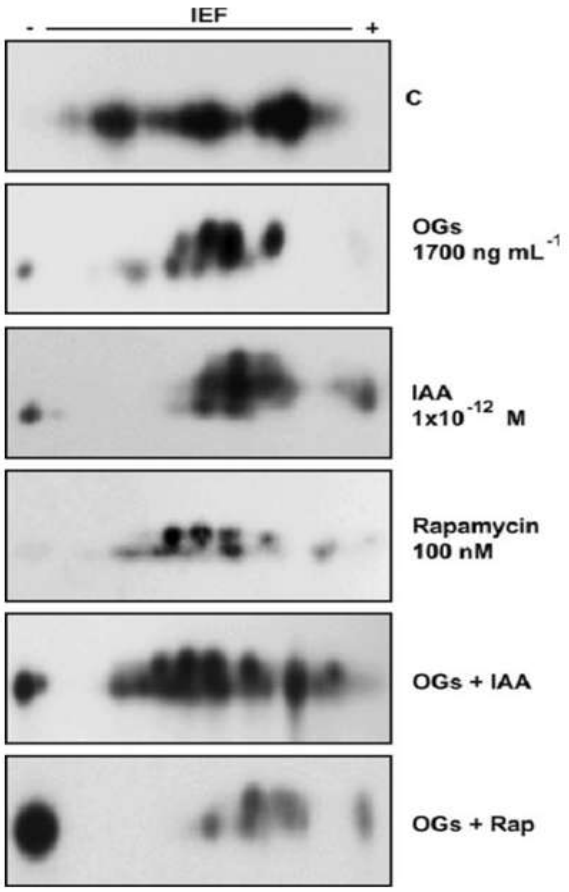


**Fig. 5** S6K 2-Dimensional electrophoretic analysis. Protein extracts from 10 days old seedlings grown in control conditions (*C*), or with indicated compounds were prepared. The combination of compounds is indicated in Fig. 4. Fifty micro grams of protein were resolved by bidimensional electrophoresis and analyzed by Western blot using anti-human p70S6K1 antibody. *Photographs* represent one of three independent experiments. *IEF* isoelectrofocusing

TOR signaling pathway in maize. During germination with a short period of OGs treatment (2 h), the S6K protein activation is detectable as an enhanced signal corresponding to an increase of protein phosphorylation. The increase in S6K phosphorylation could be mediated by free cytosolic calcium, since it has been reported an increase of this second messenger induced by short periods of OGs treatment in carrot cells (Messiaen and Van Cutsem 1994); and it has been proposed that S6K activation is dependent on interaction with $Ca^{+2}$ (Hannan et al. 2003). We used embryonic axes from 24 h germinated seeds as a model, because in the germination process metabolism reactivation, increased protein synthesis, and cell growth occurs, suggesting central role of signal transduction pathways that mediate this processes, including the TOR pathway.

On the other hand, we wonder, if this OGs mixture could modulate plant growth. After 10 days of treatment a coleoptile growth inhibition was observed with OGs 1,700 ng $mL^{-1}$, which was similar to rapamycin effect. Interestingly no additional effect in combined treatment with OGs and rapamycin was observed, suggesting that coleoptile growth inhibition was due to modulation of TOR signaling pathway by OGs. OGs induced primary root growth inhibition and lateral root formation, both effects characteristics for auxins action indicating an interaction of both compounds in different processes: growth inhibition and promotion of differentiation. Indeed, OGs-induced root formation in tobacco explants was blocked by auxins in a dose dependent manner (Bellincampi et al. 1993). However, there was no evident effect in combined treatment of OGs and IAA on coleoptile growth, which suggests that in our growth conditions this IAA concentration was not capable of restore OGs-induced coleoptile growth inhibition. Interestingly, the primary root growth inhibition induced by OGs was reverted by IAA application, indicating an inverse and tissue specific action of both compounds.

TOR signaling pathway is the master of cell growth control in eukaryotes. The scarce evidence in plants on the participation of this signaling pathway pointed out that the central role is conserved in these organisms as well (Agredano-Moreno et al. 2007; Hannan et al. 2003; Henriques et al. 2010; Menand et al. 2002; Reyes de la Cruz et al. 2004; Turck et al. 1998, 2004). In this connection, we resolved protein extract from seedlings in a 2D-electrophoresis and Western blot to correlate electrophoretic changes of S6K with the effects on growth and development shown by OGs on maize seedlings. These compounds induce a displacement of S6K immunodetected spots toward the negative pole (OGs 1,700 ng $mL^{-1}$), which could correspond to less phosphorylated protein isoforms (hypo-phosphorylated), according to that reported by Reyes de la Cruz et al. (2004); hence correlating with the OGs inhibitory effect observed on coleoptiles and primary root growth. Additionally, changes in protein weight were observed corresponding to possible post-translational modifications that might modulate S6K activity. IAA also induces changes in protein weight, in addition to a spot movement towards positive pole (IAA $1 \times 10^{-12}$ M), which correlates with the slight increase in coleoptiles growth and the increased lateral root formation. There has been reported recently that in mammals this protein is modified by ubiquitination which suggest a possible regulation through proteosome mediated turnover of S6K (Wang et al. 2008). Rapamycin induces the appearance of spots towards negative pole, even more than OGs (compare OGs 1,700 ng $mL^{-1}$ vs rapamycin 100 nM), indicating a S6K inactivation, corresponding with the growth inhibition shown by this compound in all measured parameters. This result is consistent with the maize sensibility to rapamycin shown by previous work

(Agredano-Moreno et al. 2007; Reyes de la Cruz et al. 2004) contrary to Arabidopsis insensibility (Turck et al. 1998, 2004). The combined treatment of OGs with IAA induces differential changes on S6K electrophoretic mobility (OGS + IAA), indicating an interaction of both compounds at this level. In this case both changes in the immunodetected spots occur: increased spots number indicating hypo-phosphorylated S6K and changes in molecular weight, suggesting that both compounds in combination, differentially modify this protein which correlates with the opposite physiological effects on primary root length and lateral root formation.

In summary, our result show that OGs effect on plant growth is due, at least in part, to modulation of TOR signaling pathway and that S6K could be post-translationally modified in different ways suggesting possible S6K participation in integrating growth and developmental signals in maize.

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*Estimados César Arturo Peña Uribe y Homero Reyes de la Cruz:*

Nos resulta muy grato comunicarles que el comité editorial de la Revista ***Saber más*** ha tomado la decisión de **ACEPTAR PARA PUBLICACIÓN**, su artículo **"¿Son diferentes las plantas al resto de los seres vivos?"**. Cuando éste sea publicado se le hará llegar la constancia de publicación. Agradecemos su colaboración y les invitamos a seguir participando en la revista, así como a ayudarnos a difundir ***Saber más***.

Dr. Horacio Cano Camacho
**Editor**

# ¿Son diferentes las plantas al resto de los seres vivos?

César Arturo Peña Uribe y
Homero Reyes de la Cruz

U.M.S.N.H.

Las plantas son seres vivos sésiles (que no se mueven) que muchas veces nos pasan desapercibidos como tales, para nosotros son solo alimento u ornamentos, para hacer que se vea bien nuestro jardín o nuestra casa. Pocas veces nos detenemos a pensar que mientras están ahí, imperturbables, están respirando o alimentándose, incluso podemos llegar a creer que éstas son incapaces de sufrir.

La realidad es que nosotros pocas veces logramos sentir empatía por estos seres vivos porque no se nos parecen físicamente. Sin embargo, las diferencias entre nosotros y las plantas no son tan grandes como pensamos, a nivel de procesos celulares.

## Similitudes entre genomas

Con la posibilidad de conocer el genoma (número total de genes de una especie) hemos descubierto que la "complejidad" del ser humano no es tan grande o diferente a otras especies. El genoma humano está compuesto por cerca de 30,000 genes, al igual que el ratón (también 30,000 genes, aproximadamente), mientras que el de la mosca de la fruta es de 16,000 y el de la planta *Arabidopsis thaliana* (que se usa comúnmente en investigación científica) tiene alrededor de 25,000. En tanto que el maíz tiene alrededor de 50,000 genes, muchos de ellos repetidos y no funcionales. Es decir, parece ser que el genoma del maíz es más complicado que el del ser humano.

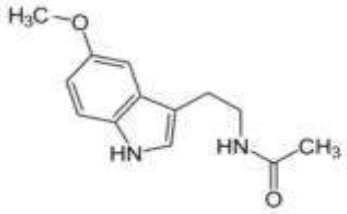
Los genes contienen la información necesaria para producir proteínas, que son los elementos funcionales dentro de las células, muchos de estos genes están presentes en varias especies (se dice que se encuentran conservados) que no necesariamente tienen relación o parentesco debido a que están involucrados en procesos celulares muy básicos, que se realizan en todas las células. Sin embargo, existen unos cuantos genes que no son esenciales para las funciones celulares y se encuentran conservados en diferentes especies.

Existen diferentes tipos de genes que se encuentran tanto en el humano como en las plantas. Algunos de ellos esenciales para algunos procesos celulares vitales y otros tantos que nos provocan curiosidad por su presencia en los vegetales. Como mencionamos anteriormente, los genes producen proteínas que a su vez tienen una función en la célula, como puede ser el generar compuestos que provocan diferentes efectos en las células, son estos productos los que en realidad llama la atención encontrarlos en las plantas y que llevan a cabo funciones muy particulares en el ser humano.

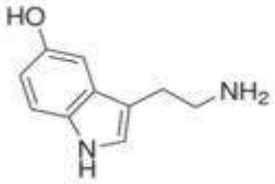
*Arabidopsis thaliana: Vijay Singh*

## ¿Melatonina, serotonina e insulina en plantas?

En años recientes se ha reportado la presencia de melatonina y serotonina en plantas. La melatonina en animales regula los procesos del sueño, la percepción de ciclo diurno/nocturno mientras en plantas regula procesos de desarrollo y crecimiento vegetal. La serotonina es un neurotransmisor que en animales regula el estado de ánimo, los cambios de humor y algunos procesos cardiovasculares. En plantas, este compuesto regula varios aspectos del desarrollo de la planta como son la floración, como protector antioxidante y más importante aún como regulador del crecimiento vegetal. Estos compuestos presentes en plantas nos hablan sobre las similitudes a nivel biológico que hay entre nosotros.




Melatonina




Serotonina

Otro ejemplo sobre este tipo de compuestos es la insulina, recientemente se ha reportado que el maíz produce una pequeña proteína (péptido) muy parecida a la insulina humana, este péptido del maíz es incluso capaz de funcionar como insulina humana en células adiposas de humano, permitiendo la entrada de glucosa a los adipocitos. En otros aspectos, este péptido induce el crecimiento celular de la misma manera que lo hace la insulina humana.

Insulina humana

## Mecanismos de comunicación en plantas

A demás de todo lo mencionado, las plantas tienen sus propios mecanismos de percepción y respuesta a estímulos ambientales. Dada su característica sésil, las plantas han evolucionado para responder a las adversidades ambientales que les rodean. Si un insecto trata de alimentarse de ciertas plantas, éstas son capaces de producir toxinas para defenderse. Otro mecanismo de supervivencia es la producción de compuestos que le sirven de comunicación química entre las mismas plantas e incluso les sirven para comunicarse con bacterias y hongos que pueden ser benéficos para su crecimiento.



¿Son diferentes las plantas al resto de los seres vivos?

Es interesante también, saber que cuando se arranca una hoja o una rama a una planta, en ésta se desencadenan mecanismos de reparación y de defensa, para proteger la zona de posibles depredadores.

Todos estos hechos indican que las plantas son capaces de percibir y reaccionar a diferentes situaciones de su entorno, que a su manera, se presentan en respuesta a lo que les hacemos y como las cultivamos. De llamar la atención son esos compuestos que creíamos propios de animales, que las plantas también utilizan para regular aspectos de la vida y desarrollo vegetal, además de que su respuesta a estímulos ambientales puede ser tanto o más compleja que la de los humanos y nos hace decir que: las plantas se nos parecen, en ciertos aspectos, más de lo que podríamos imaginar.

**Para Saber Más:**

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¿Son diferentes las plantas al resto de los seres vivos?

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